The role of developmental feedback between insects and fungi in wood decomposition processes

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THE ROLE OF DEVELOPMENTAL FEEDBACK BETWEEN INSECTS AND FUNGI IN WOOD DECOMPOSITION PROCESSES

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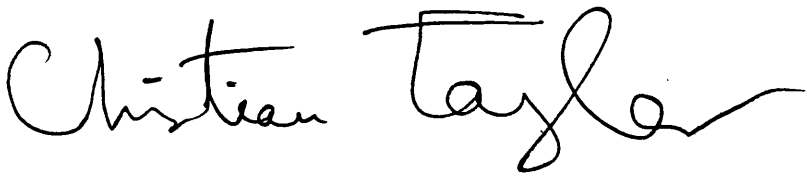

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Christian Taylor
2001

To lichens, whose symbiosis, the interactive interplay between algal and fungal form, provides a metaphor for living complexity's co-existent couplings. Here, as with prokaryotic endo-symbiosis and bio-film anabolism, lie stories of lasting synergies of two or more entwined forms, enhancing potential for chemical and physical morphogenesis to emerge from their interactive states, which were neither of one nor the other, but which were induced from relationship - the fluid, reciprocal-influence between things that evolve.

And to my parents Gitta and Anthony; for my childhood in a wooded, wild and wet Cornish valley, and for letting me learn through experience.

"Here there was more space between the trees and many bushes in the spaces. They came presently to a clearing that a great tree had made before it died, a clearing close by a river and still dominated by the standing corpse of the tree. Ivy had taken over, its embedded stems making a varicose entanglement on the old trunk and ending where the trunk had branched in a huge nest of dark green leaves. Fungi had battened too, plates that stuck out and were full of rain-water, smaller jelly-like blobs of red and yellow so that the old tree was dissolving into dust and white pulp. Nil took food for Liku and Lok pried with his fingers for the white grubs."

(From : The Inheritors, by William Golding, 1955.)

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Abstract

Interactions between saprotrophic fungi, insects and wood influenced decomposition via developmental feedback. Multiple-scaled investigations of insect-fungal community composition and distribution in a hazel coppice revealed mycophagous, paedogenetic, dipteran, *Cecidomyiidae* larvae to inhabit narrow sub-cortical niches of dead wood spalted by pseudosclerotial plate (PSP) between adjacent fungal decay columns. During PSP development, reciprocal metabolic exchange between mycelia was correlated with enhanced development of sub-cortical cavities into a heterogeneous array of interconnected micro-tree holes under bark. Here larvae were shown to assort themselves according to body size. Cavity-size varied according to temporal insect-fungal succession, tree species and bark thickness. Interaction experiments paired fungal mycelia of ascomycete *Hypoxylon fuscum* and basidiomycete *Vuilleminia commedens* with *Brittenia fraxinicola* cecid larvae. Developing larvae affected redox-chemistry, increased pigmentation and PSP thickness. In turn, cecids were affected by mycelial development. Larvae exhibited changed life-cycle duration, progeny number, body size, foraging pattern, diapause and birth-place choices. PSP was characterised by altered catalase and peroxidase activities and altered levels of antioxidant and hydrogen peroxide. I suggest such changes influence larval development patterns. Multipartite experiments suggested that *H. fuscum* and *B. fraxinicola* might be facultatively mutually symbiotic via negative indirect effects on *V. commedens,* reducing its combative ability. Also larvae may have faced a nutrition/refuge trade-off whereby their PSP refuges from oxidative-stress appeared to be lacking food whilst active food hyphae were associated with free radicals. The woody environment in which larvae and mycelium co-inhabit was correlated with reduced wood hardness and increased cavity emergence between intact medullary rays. A schema is discussed whereby fractal cavity-architecture alleviates competitive exclusion, increases co-existence via resource partitioning and allocates chemical refuge from oxidative-stress. It is suggested that biochemical synergistic effects seed physically synergetic emergence of rot-hole architecture through feedback. This interactive-coupling process links co-existence to generation of surface area, heterogeneity, symbiosis and emergent morphogenesis.

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CHAPTER 1: FUNGI AND INSECTS INSIDE DEAD WOOD - CONCEPTS OF DEVELOPMENT, CONTEXT AND FEEDBACK

1.0 Synopsis

This study considers the process of feedback between interacting living systems as the catalyst of ecological relationships. It investigates development patterns through which feedback may be recognised, and suggests how feedback processes couple small to large-scale ecological interactions. Understanding how living feedback systems combine at multiple-scales has application both to theoretical and conservation biology. Also it can better enable us to answer important contemporary questions such as how so many competing species have evolved to co-exist in such close proximity and how do patterns of co-habitation and behaviour emerge from a developmental and structural point of view? This work incorporates a range of woodland species from three taxa local to Bath, UK, and asks principally how they relate. Species of wood-decay fungi, insects and tree exemplify intra- and inter-specific as well as taxonomic interactions. This chapter introduces those aspects of the biology of wood-decay fungi, trees and insects that I hope show a likelihood of developing through a process of feedback with their surrounding biotic and abiotic milieu. As far as I am aware, this project has been the first to investigate reciprocal developmental interactivity between insects and fungal inhabitants of decomposing wood. This project is the first to examine inter-specific interactions between saprotrophic fungi of different sub-divisions using a multi-scaled range of both field and biochemical techniques.

1.1 Wood-decay Fungi

Fungi connect a large variety of interactions between taxa and niches in forest ecosystems. They form links in the relationships that enable ecosystems to occur (Pirozynski & Hawksworth 1988). Fungal mycelia are fundamentally involved in the processes that form patterns in most types of terrestrial and many marine and benthic ecosystems. Studying the interactive interfaces that define fungal relationships with other taxa enables ecological pattern-forming processes to be understood (Hedger 1985). Relationships consist of feedback loops of reciprocally exchanged entities, which simultaneously change both parties and create new synergies. Understanding the mechanism of such feedback loops could enhance present explanations as to how feedback communication processes create ecosystem patterns, both from the distal (evolutionary) and proximal (ecological) standpoints.

Insects and fungi together make up approximately half of the estimated 100 million biological species that inhabit planet Earth (Swerdlow & Morelle 1999, Wilson 1992 a, Egorova 1998, Hawksworth 1998, Siepel 1996). Fungi inhabiting dead wood perform at least two fundamental macro-ecological processes that may influence biodiversification. These processes, without which forests would not exist (Wilson 1992 a, Carrol 1995, Harley 1971, Heal & Anderson 1979, Ingold & Hudson 1984), include lignin decomposition (Blanchette 1995), cellulose decomposition (Cooke 1984) and the consequential re-cycling of forest carbon and nitrogen (Flemming 1993, Dighton 1995). These processes influence local conditions in which they

proceed, affecting environmental factors such as water availability, humidity, temperature, pH and aeration, in which other species inhabit (Boddy 1983, 1984, Boddy & Watkinson 1995). It has been shown that fungal growth and succession affects abiotic factor fluctuations amongst roots, sand and soil, and is important in providing a means to plant-fungal community succession (Frankland 1998). Also fungal communities have been implicated in dampening what would otherwise be dramatic abiotic factor fluctuations within sand dune ecosystems. Likewise, wood-decay fungi may affect oscillations of abiotic factor fluctuations in forest ecosystems (Schmiedeknecht 1998, Dighton 1997). For example, fungal decomposition is known to produce catabolic heat during decay in compost and silage processes. However, this ability has not yet been demonstrated in forests perhaps because of difficulties in measurement. Woodland decay activities are both less rapid and less concentrated and so are more ephemerally distributed than when involved in artificial silage and composting. However, there is no reason to expect that fungi only produce heat in artificial circumstances (Augustin 1998), especially since the fungal communities in silage and compost are often seeded from natural surroundings. Thus it seems reasonable to suggest that fungi generally effect abiotic factor fluctuations around non-fungal organisms that inhabit them, especially regarding water availability, temperature and pH. An organism may survive abiotic stress by orientating itself in a place of fungal activity, for example a piece of rotting wood where chemical and physical conditions do not fluctuate outside limits in which a species can survive. This is particularly relevant for hibernation and drought survival for many invertebrates including insects. It is from this later perspective that fungi may be considered dampers of abiotic factor fluctuations and as imparting some degrees of stability to a decomposing woody niche.

However, over longer-scaled spans of time of months and years rather than days and weeks, abiotic conditions have been shown to be in constant flux and are also spatially dynamic, unstable and heterogeneous in a forest (Crawford 1989, Taylor 1994, Rayner 1997). Regarding these longer time periods, fungi may contribute to spatial and temporal shifts in the microclimate as they dynamically explore and grow through a forest (Cooke & Rayner 1984, Griffith & Boddy 1991). Water availability (Rayner 1999), humidity, pH and secondary metabolites (Frankland 1998, Gloer 1995) have been implicated in their involvement in biochemical oxidative stress, as manifested in the fungal protoplasm (Watkins 1998, Watkins, Beeching & Rayner 1998 a, b) as in all cellular life (Georgiou 1997, Fee 1980, Oppenheimer & Stern 1939). It has been further suggested that levels of such protoplasmic stress contribute to shifts in distribution patterns of many fungal developments within a forest from scales of mm to Km (Frankland 1998, Rayner 1997, Wildman 1998).

So fungi contribute to the maintenance of niche conditions for short periods of time up to weeks (Porati, Granero 2000), but on the other hand contribute to spatial niche-shift over months and years (Pool 1989). Fungal activities that bring about the maturing, ageing, senescence and deterioration of niche conditions, are complemented by those that simultaneously lead to the fresh emergence of that niche in other places (Boddy, Wellins & Lyon 1988, Rayner 1993 a, b). As Rayner has metaphorically put it: "Fungi may be considered the Brahma and Shiva of the forest" in reference to Hindu texts where Brahma is the first of the three "Trimurti" and the creator of life and conditions in which it flourishes. Shiva is the third of the Trimurti and energetic destroyer - who sweeps away the old to make way for new emergence. The other Trimurti is Vishnu: preserver, conserver and protector (The Bhagavad-Gita), whose important ecological roles are arguably also achieved by fungal activity (Rosenberg 1974, Rayner 1992 c, 1993 a, b).

Such dramatic fungal activity clearly affects the biology of many other species (Cloudsley-Thompson 1964, Mongold, Bennet & Lenski 1996). For example, if an organism currently situated in an isolated fragment of its niche is to grow in a newly emergent fragment elsewhere, it must first disperse and locate this new site, involving either growth, exploration, or both, via asexual or sexual reproduction or a combination of each. Those parts which remain in the old fragment may enter dormancy until niche conditions might improve. Dormancy or dispersal may be triggered by niche senescence and form key components of insect and fungal life-cycles. It is within these developments that species may temporarily exist inside highly insulated states, for example spores and pupae, to protect from oxidative or free radical induced stress (Rayner 1998 b, Ramsdale 1996, Watkins 1998). Such developmental stages within life-cycles are followed by germination, breaking symmetry (Rosen 1989, Chernikov *et al.* 1989, Lengyel 1989, Senior 1989, Shakunle 1998) or hatching processes that coincide with experience of fresh niche conditions when life can continue with thinner insulation (Rayner 1997, Yazdani & Agarwal 1997). In light of this it seems reasonable to suggest that changes in fungal states might provide some of the environmental cues used by non-fungal organisms such as insect larvae to enter new developmental stages, so effecting how these organisms progress through their life-cycles. This leads to the following question: **"How much is the progression which forest organisms make through their life-cycles being partially determined by fungi in their local context?"** In temperate forests, seasonal changes and microclimate will undoubtedly alter fungal developmental states (Boddy 1983, 1984, 1986), so it becomes hard to distinguish those dynamics caused by the climate from those produced by fungi. However, in some tropical forest habitats where seasonal changes are more subtle, it may be fungal activities that supersede the effect of climate or water availability in bringing about dramatic changes in niche conditions in a particular patch of forest habitat.

In addition to spatial niche-shift, fungi may also contribute to and sometimes induce temporal developmental change (Guevara 1997) within a particular niche, from freshly created, to mature and senescent (Gardes 1998, Whittaker & Levin 1975). The fungus *Coriolus versicolor* has been shown to provide expansive arenas that enabled the temporal resource partitioning of two species of small ciid beetle (Guevara 1998). Here the term "Dynamic niche" (Guevara, Rayner & Reynolds 2000) was applied to changing niche conditions (Glauert 1960) of bracket fungal fruiting bodies of *Coriolus versicolor* and resident insect beetle species that were partitioned by the different fungal shapes and volumes emerging from a decaying log. Another example, at much greater spatial scale, is that of mycorrhizal relations which have been shown to affect the type of symbiosis between tree species in forests (Fogarty & Requad 1998). This in turn implies that many niche boundaries are perhaps themselves the shifting, branching, stretching and folding surfaces of growing and developing organisms (Frank & Amaraskare 1998, Thom 1992, Straw 1994, Wallinga 1995).

So fungi play a fundamental ecological role (Rayner 1993 a, b) in developing, maintaining and destroying niche space in forests (Guevara 1998). Knowledge of how fungi interconnect the lives of many invertebrates to their niche spaces will enrich understanding of forest dynamics and also greatly aid our attempts to conserve biodiversity (Givnish 1999).

Leaving aside the large ecological effects of fungi, let us now look at fungal diversity (Figure 1.1). The fungi form a macro-diverse kingdom of their own (Wilson 1992 a) of which science has discovered about 76,500 fungal species, as extrapolated to 2000 from Hawksworth's paper (Hawksworth 1990), with about 600 species new to science discovered annually (Frohlich, Hyde 1998, Franco-Molano 1998). The global

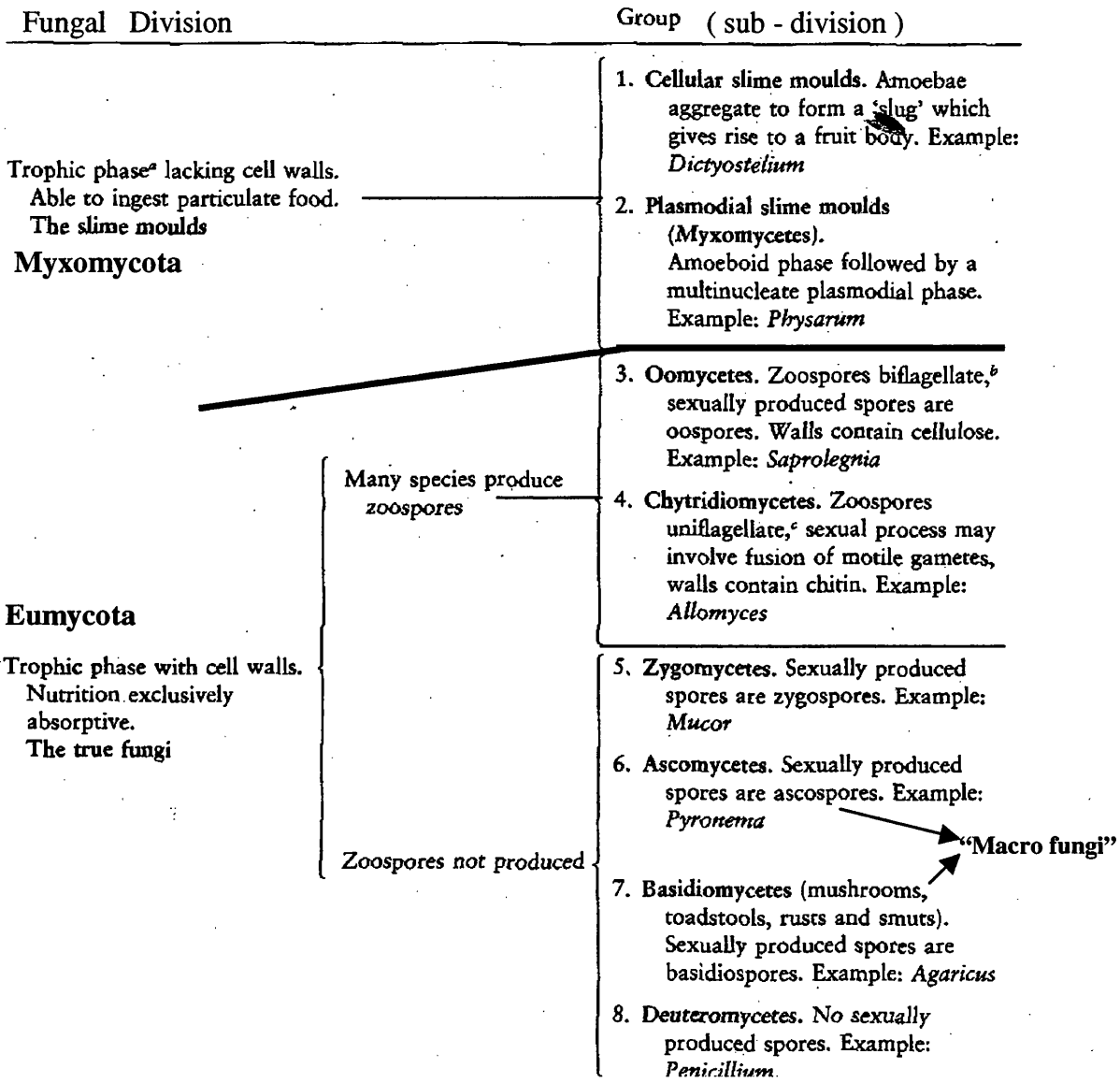


Figure 1.1 Eight fungal groups (sub-divisions) into which species of the fungal kingdom are classified. To date only a small fraction of the fungi have been classified with several new species to science appearing in every mycological research publication, especially from tropical regions. Basidiomycetes and ascomycetes represent the fungi which have the most visible forms in ecosystems generally, although deuteromycetes and zygomycetes may be very common. A yeast is a unicellular budding phase which may belong, potentially, to any of the Eumycota. *Sacchromyces cerevisiae* happens to be an ascomycete yeast. In aqueous environments, many fungi undergo developmental shifts and produce yeast phases.
(from Carlie and Watkinson 1995)

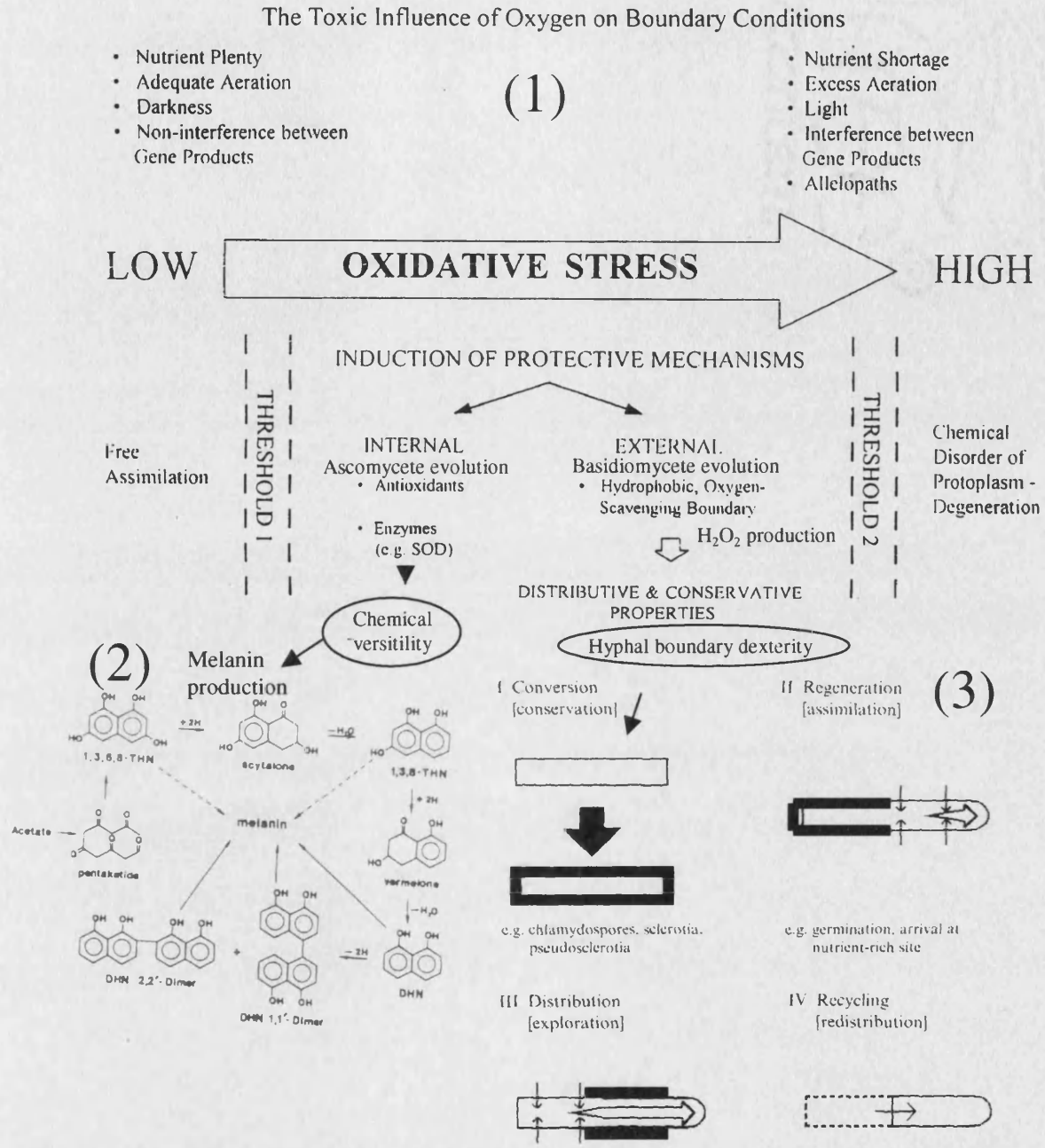


Figure 1.2 (1) shows the relationship between oxidative stress and the two strategies of protecting hyphal protoplasmic boundaries with internal or external methods (Rayner 1996).

2 shows consequences of chemical versatility of the ascomycetes; production of anti-oxidant chemicals, enzymes and pigments such as fungal melanins (Adapted from Henson *et al.*1999).

3 shows consequences of hyphal boundary dexterity of basidiomycetes - the insulation thickening of hyphal walls to protect from free radical stress results in greater throughput and emergence of diverse of mycelial growth forms (from Rayner 1996).

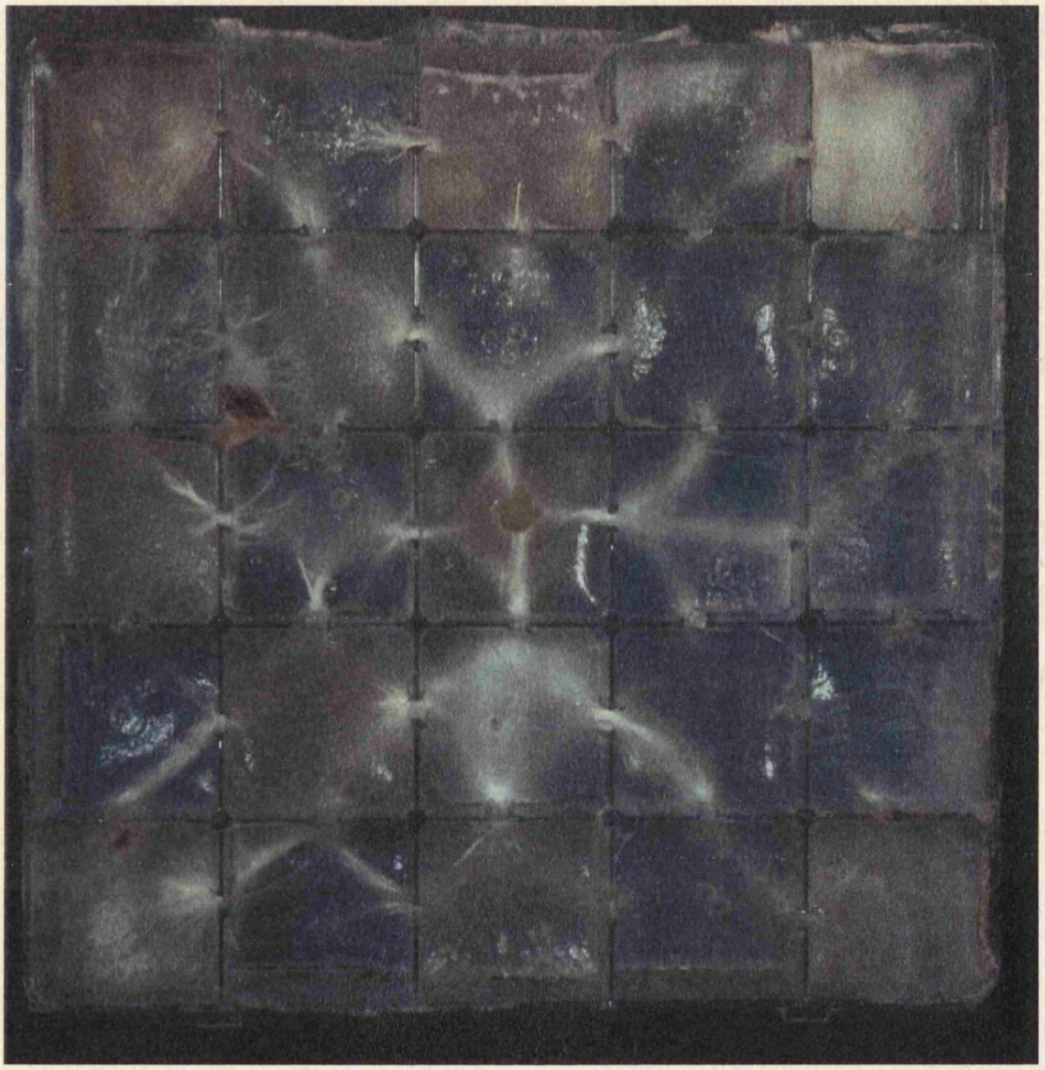


Figure 1.3 Mycelial dexterity of basidiomycetes; In this case *Phanaerochaete velutina* exhibits it's potential range of hyphal wall insulation attunements to a heterogeneous chequerboard array of inter-connected high and low nutrient domains of a repli-plate. The high nutrient wells are slightly beige in colour due to the presence of thin-walled dense and slow growing, assimilative mycelium and the catabolic enzymes released extra-cellularly . The clearer coloured wells which lack such pigmentation have low nutrient supply. The basidiomycete mycelium which crosses these domains is highly impermeable, insulated with thickened hyphal-wall ultra structure. This explains why mycelium in these low nutrient domains possesses an enhanced ability to reflect light.

estimate of number of fungal species ranges from 1.5 million to 13 million (Cannon 1998, Hawksworth 1991, Jancovicova 1998) so as much as 5.1 % to as little as 0.58 % of the kingdom is formally known to science. This makes fungi the second most numerous eukaryotic group on the planet after insects, yet mycologists have intimately studied the biology of only about 100 species, of which one, the yeast *Sachromyces cereviciae*, has had its genome fully sequenced, mutated and thereby mapped. About fungi there is thus the largest amount of "unknown" in proportion to any eukaryotic taxon (Wilson 1992 a, Hawksworth 1991). Fungal biology is thus fascinating not only because it is ecologically fundamental to our understanding of life on earth but also because there is so much new to discover to enrich the science of biology as a whole (Hawksworth 1998, 1990).

As shown in figure 1.1, there are two fungal divisions (phylum equivalents), the true fungi or Eumycota, and the slime moulds or Myxomycota. These divisions are sub-divided into eight fungal sub-divisions (equivalent to sub-phyla), which can also be seen in the figure. Two sub-divisions with which this work is principally concerned, Ascomycotina and Basidiomycotina, can be referred to as "macro-fungi", with easily seen structures. These have traditionally been classified morphologically looking at spores, fruiting body characteristics and habitat. However, it has been suggested in the works of Rayner and Watkins that Basidiomycetes and Ascomycetes may also be distinguished in terms of their adaptive strategies to reduce oxidative stress (Watkins, Beeching, Rayner 1998 a, b, Cooke & Rayner 1984). This basis for this idea, shown in figure 1.2, is strengthened by mitochondrial ultrastructural divergences between fungal groups, and the fact that dysfunctional mitochondrial receptivity of oxidative stress-induced signals from the cytoplasm forms the basis of both incompatible mating and inter-specific interactions (Fee 1980, Gloer 1995). The communication between mitochondria and two nuclei in each hyphal compartment, together with signals generated in the protoplasm, for example in peroxisomes, are thought to effect protoplasm secondary compounds, through polyphenol metabolism, and hyphal wall chemistry, via hydrophobin metabolism. This in turn affects the physical growth and development of fungi according to major taxonomic groups. Such events mirror a parallel biochemical theory for appreciating the present classification of plants in terms of secondary plant metabolites such as latex, tannins, coumarins, and flavenoids (D'Auzac 1996). A development shift that many fungi can induce under certain conditions is a yeast-hyphal dimorphism, exemplified most often by Basidiomycetes and Ascomycetes (Prillinger 1986).

Six remaining fungal sub-divisions in figure 1.1 are referred to as "lower fungi" (Hawksworth 1998), most of which are also "micro-fungi", not seen easily without a hand lens. They do not have the ability to negotiate large spans (centimetres to kilometres) of heterogeneous landscape as their "higher" counterparts do, but at much finer scales (Read 1990), of micrometres to millimetres these fungi use a combination of albeit limited chemical versatility (Hawksworth 1987, Crowe 1998, Pieckova & Jesenska 1998) and hyphal boundary dexterity to survive (Rayner 1999) (figure 1.2). They often form spores on ends of microscopic stalks such as the Zygomycetes, of which Mucorales are an order, and Deuteromycetes, of which *Penicillium* is a genus (Carlile & Watkinson 1994). One of these six remaining sub-divisions, the Myxomycetes, however, are plasmodial slime moulds which alternate between collective foraging networks (Rayner 1997, Carlile & Watkinson 1994) with dispersal of spores when conditions are stressful, and singular independent foraging as amoebae when conditions are plentiful (Rayner, Watkins & Beeching 1999). Generally, the propensity for all these fungal groups to juggle their varying abilities of chemical and hyphal performance (Wicklow 1988, Williams 1981 a, b) could perhaps be viewed as an emergent property from feedback between their inner

protoplasmic environments and outer inter-cellular contexts, as distinguished from and influenced by surrounding environmental conditions (Rayner 1997, 1998 a, Jordanova 1984, Pigliucci 1996).

1.2 Developmental feedback in natural populations and communities of wood-decaying fungi

Growth and development are different but highly inter-linked processes (figure 1.3) (Sussman 1964). Growth usually entails the expansion of mass and spatial volume of an organism. However, such size increase alone may produce a number of difficulties that must be overcome through development (Stanton, Roy & Thiede 2000). Indeed, broadly speaking, it is the degree to which evolution has equipped species to develop topologically by directing the local instabilities induced by chemical and physical interactivity (Thom 1972) into stretching and folding boundaries, tissue layers and cell-types which enables organisms to survive being both large-sized and metazoan (Thom 1992). This is not to be taken that large-scaled organisms are necessarily evolutionarily more advanced (Haldane 1985, Smil 2000), size is just one of potentially infinite criteria which could select in any direction (Bonner 1965). However, when growth of body size does confer an adaptive benefit in a particular environment, as larger individuals are selected from a population, so too are abilities to develop, i.e. to produce phenotypes and genotypes that are adapted to being large sized (Haldane 1985). So organisms are perpetually attuning their development, through natural selection, to the environmental challenges of their niche space.

Mutual interactivity between biotic environments produces pattern via reciprocal developmental feedback across boundaries (figure 1.4). This is well exemplified by branching patterns of plants (Harper 1986) and hyphae of woodland fungi (Butler 1984). Fungi possess abilities to form higher-order **intra-specific mycelial boundaries of somatic rejection** when different mycelia of one species meet in soil or wood and mate unsuccessfully. Somatic rejection, a form of hyphal boundary dexterity, occurs where hyphal-lysis is induced by oxidative-stress and heat-shock through metabolic dysfunction between incompatible mitochondria after plasmogamy between mycelial genets. The process is analogous to apoptosis. Also the formation of antagonistic **inter-specific responses often result in another type of higher order boundary, that of pseudosclerotial plate (PSP)** between mycelial domains. PSP is a polyphenolic hydrophobin-rich melanised zone of lysed hyphae emergent from secondary compound interference. PSP production changes the environment in which a fungal community is structured, altering the potential development of future patterns of fungal succession. PSP, once formed, is highly persistent and durable physical structure (Georgiou 1997). Such reciprocal developmental feedback is exhibited at many scales throughout fungal systems and their ecological assemblages (Rayner 1998 a, Rayner *et al.* 1999 a).

Fungal hyphae (Figure 1.5), being relatively un-fixed and correspondingly genetically soft-wired (Rayner *et al.* 1985), alter their development according to changes in contextual information which they sense (Rayner 2000). This is thought to be in the form of signalling between and inside hyphae as well as receptivity to environmental dynamics (Rayner, Ramsdale & Watkins 1995). Since fungi influence environmental conditions profoundly through their activities (Rayner 1993 a), this too can be viewed as developmental feedback (Tsuneda & Thorn 1995).

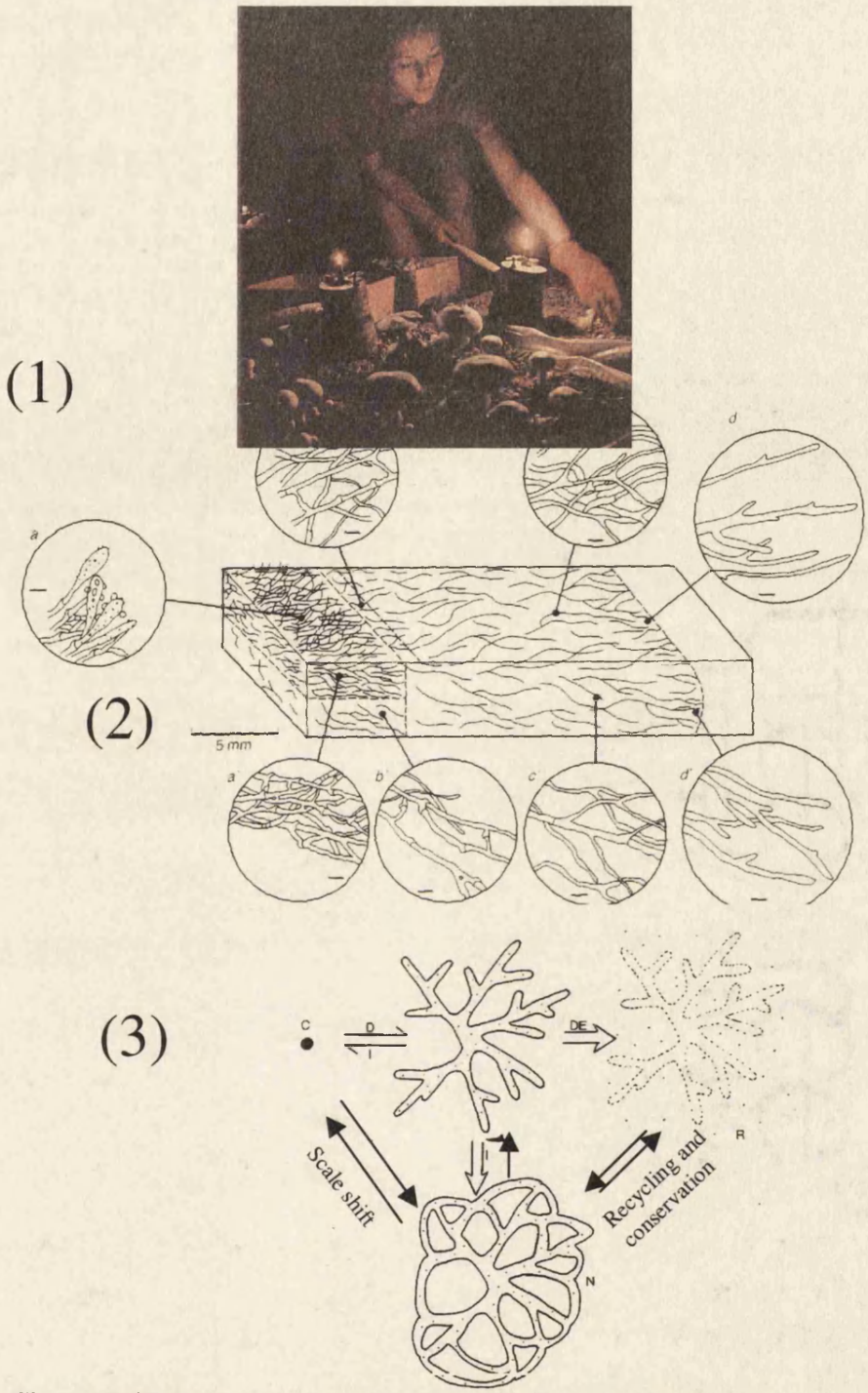
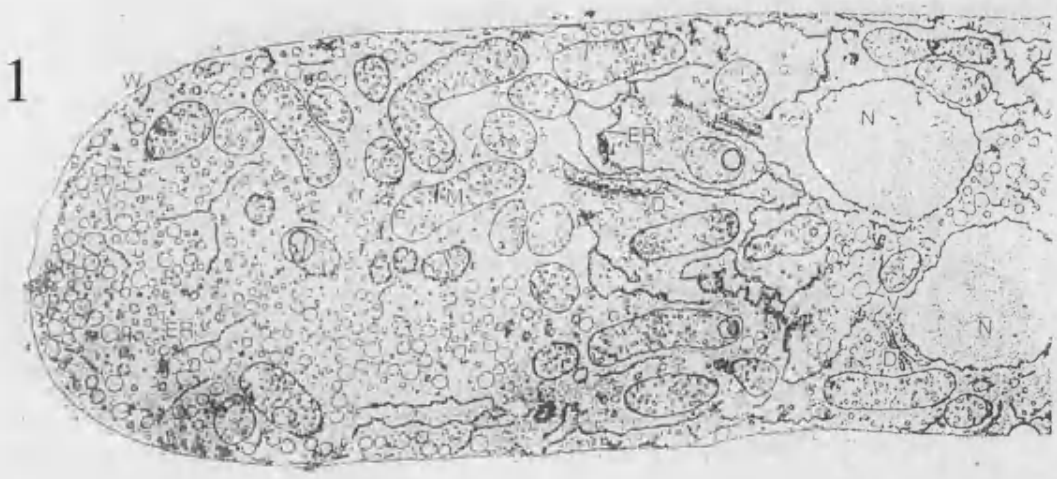
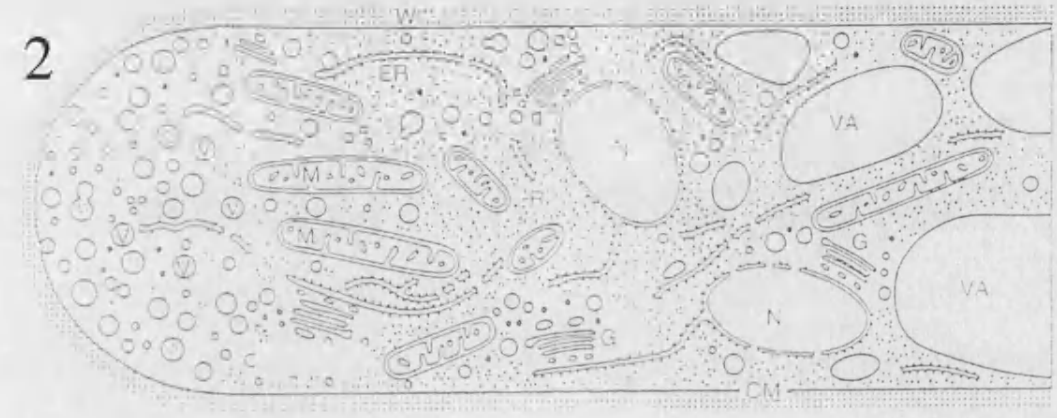


Figure 1.4 Below basidiomycete fruting bodies of *Agaricus bisporus* (1) lies the developmental feedback of fungal mycelium within a decomposing environment (2). (2) Shows a schematic of an ageing mycelium from right (young) to old (left). Illustrated below (3) are 5 types of developmental shift in fungi germination and assimilative growth to senescence, recycling or anastomosis.

(2) shows colony characteristics of a basidiomycete growing through malt agar towards the right. The colony margin (right) is less anastomosed (networked) and more assimilative than the older (left) highly anastomosed mycelium which possesses greater distributive and reproductive potential. (from Body and Rayner 1983).

(3) shows development of a fungus from spore to divergent asymetry and then either senescence (right) or networking (anastomosis) beneath. The structure below could be regarded as equivalent to a spore (ie a survival structure from which asymetric emergence may re-occur); so representing a change in scale.





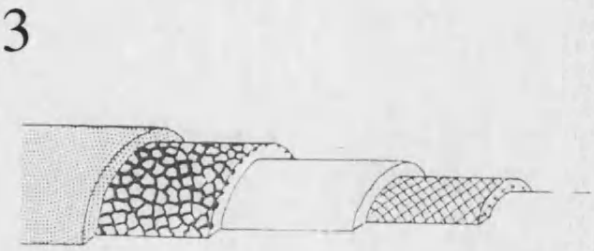
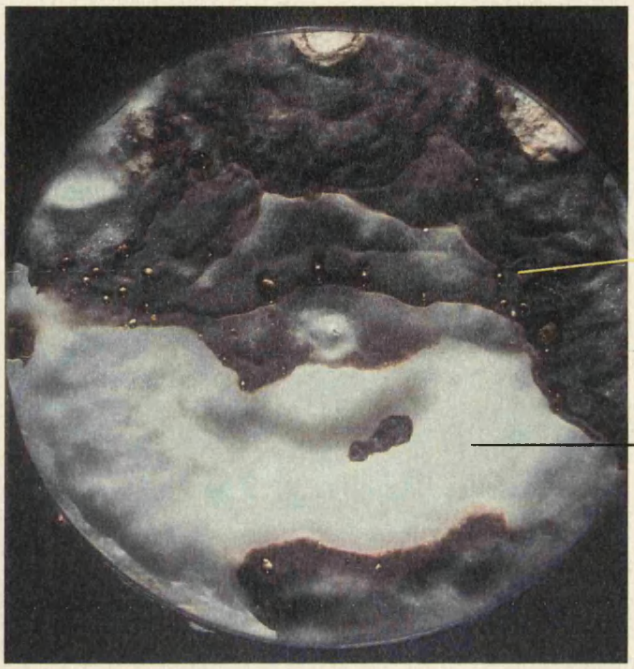


Figure 1.5 Nested and multiply enfolded phospholipid bilayers with proteins which control membrane permeability (1) which make up the living hyphal protoplasm. This protoplasm is shown schematically at middle with plastids and other components of hyphal ultrastructure (2). (3) shows, in greater detail, what surrounds, protects and directs the flow of hyphal protoplasm; up to 4 layers of hyphal wall insulation which can be thickened or broken down according to feedback across underlying membranes. (from Carlie and Watkinson 1995).



Psudo sclerotial plate (PSP) between *Corticium evolvens* and *V. commedens*

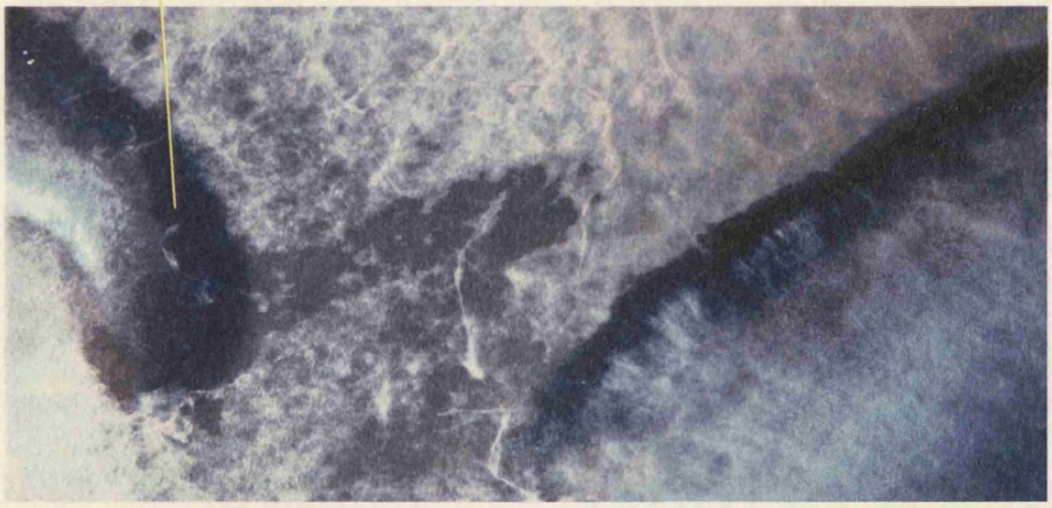


Figure 1.6 Top shows a sclerotial blanket covering stressed regions of the mycelium of a pure culture of the fungus *Hymenochaete corrugata.* The blanket forms at the boundary between stressed, scenescing mycelium and the outside world. This protective envelope; the product of redox chemistry equivalent to melanisation, can also be produced between different species; as pseudo sclerotial plate (PSP) shown in the lower image. Chemical and physical properties of such boundaries can differ greatly from those within mycelia on either side, therefore increasing diversity; a theme which will be one of the main to be enlarged during this thesis.

Fungi are perhaps particularly susceptible to experiencing dynamic conditions (Rayner 1991, Rayner, Boddy & Dowson 1987, Rayner & Coates 1987, Rayner, Watling & Frankland 1985, Rayner 1993 a, b). Unlike other organisms which protect their tissues within tough envelopes in which relative homeostasis may occur, fungal hyphae grow through the very substrates on which they feed, digesting and assimilating them as they proceed (Rayner, Watling & Frankland 1985). Not only are these substrates often soil or dead parts of plants and trees subject to abiotic environmental fluctuations (Coats & Rayner 1985, 1987), but also these substrates are doubly-dynamic in the case of fungi because of the process of decomposition (Boddy & Dowson 1987). Fungi digest their own homes. Dynamic changes in fungal substrates, which might otherwise kill hyphae if they were too genetically fixed, are countered with a dynamic, unfixed and indeterminate development with correspondingly fluid genetic regulation (Spitze & Sadler 1996, Ho 1993) which enables fungal mycelia to thrive in changing circumstances (Boddy 1986, 1992, Coats, Rayner & Boddy 1985) and ramify into their food sources with a phenomenal surface area to volume ratio. Research has indicated that similar processes in plants (Smith 1999, Walbolt & Cullis 1985, Wijesinghe & Hutchings 1997) relate their assimilative surface area to indeterminacy and heterogeneous asymmetry.

From now on I shall concentrate on how the two sub-divisions of "macro-fungi", Basidiomycetes and Ascomycetes, adapt their developments to changes in environmental conditions (Figure 1.6). Information regarding environmental dynamics (Lyklema 2000) is relayed through a complex of often greatly enfolded semi-permeable boundaries and spaces (Rayner & Todd 1982 a, b, Rayner 1989 a, b) via a series of coupled cyclic metabolic equilibria that occur in feedback directly or mediated indirectly by secondary messenger signals (McClintock 1953, Baskin & Norde 2000, Kleijn & Leeuwem 2000). This fungal communication eventually causes changes in the developmental fate of portions of collections of hyphae or organelles within a portion of a single hypha (Latchman 1990, Kirchner, Gerhart & Mitchison 2000). Signals may change the nature of incoming information via feedback processes (Lyklema 2000) by translating from the original source to a second molecular language (Kordon 1993). Thus the original nature of a message, for example an increase in humidity around a log at nightfall, is passed first through envelopes of protective dead matted-hyphal fans under bark (Ramsdale & Rayner 1997), then translated into the effects humidity increase has on this matted fabric. For example, gaps in the fabric enlarge between hyphae to the point that water may seep through instead of being repelled by the wall's hydrophobic matrix components that hitherto have prevented water loss from the underlying mycelium (Unestam 1991). The original message's effects have now entered a microcosm (Bertreaud & Alfsten 1976), the space between the aerial mycelial covering and sapwood in which are found assimilative hyphae of the underlying fungal mycelium. A new feedback cycle then picks up from where the former left off and relays the message as an increase in water availability between individual hyphae across hyphal cell wall fabrics (McNiven *et al.* 2000) to the membrane and its constituent proteins underneath (Rodger 2000, Baskin Norde 2000, Baror 2000). A further feedback cycle then affects pinocytosis via the clatherin coatings on the protoplasm side of the membrane. Depending on whether a hypha is flaccid or turgid and its osmotic pressure (Pelczar, Chan & Krieg 1986), it will either take up water surrounding it or not. In taking up water, a number of developments may concur such as growth and elongation of hyphal tips, reproduction of organelles, mitosis, and protoplasmic streaming from areas of low to high water potential. It has been indicated that the extent to which yeast walls alter their permeability involves changes in the conformation and binding sites of proteins (Rodger 2000) and chitin in the poly-saccharide wall insulating a hyphal membrane (Kapteyn *et al.* 1998, Klis 1998, Nutsubidze *et al.* 1998). It is further indicated from yeast cell cycle mutants lacking ability to develop through each cycle that a loss of

ability to alter cell wall fabrics in response to different stages in the cell cycle confers their mutancy (Klis 1998). This strengthens the suggestions by Watkins, Beeching & Rayner that mycelial developmental instability could be attributed to their abilities to control cell wall and membrane permeability and reduce accumulation of senescent and ageing-accelerating damage rendered by superoxide-free radicals and reactive oxygen intermediates (ROI) (Hansberg & Aguirre 1990). The process of hyphal insulation is augmented by production of either chemical or enzymatic anti-ageing antioxidants and other free radical mopping enzymes in response to local heterogeneous environmental conditions (Watkins 1998, Rayner 1997, Yu 1993).

Feedback processes curtailing oxidative stress (Baror 2000) within hyphal protoplasm can be categorised into three levels as follows. When an input of stressful conditions requires a change of insulation around a hypha, the cell wall first responds by expressing an altered conformation and binding of protein and chitin molecules already present in the cell wall fabric (Bart-Nicki Garcia 1987, Gooday 1992, 1979). This first-degree response is emergent from the changed conditions themselves which alter the redox chemistry of molecules at the wall matrix (Bart-Nicki Garcia, Ruix-Herrera & Bracker 1979). It has been suggested that such changes be termed hyper-epigenetic (Rayner, Watkins & Beeching 1999). However, if this response fails to limit the stress such as water loss or a perforation of protoplasmic membrane, the stress filters through to the underlying protoplasm. Here a second-degree or epigenetic (Rayner, Beeching & Watkins 1999, Rayner *et al.* 1999 c, Lewontin 1994) response alters the metabolic activity of enzymes and chemical antioxidants within the protoplasm. So far the changes have all been responses to environmental and epigenetic signals (Baskin & Norde 2000) that have not necessarily required any recourse to transcribe genetic codes (Gorovits *et al.* 1998). Such events mirror developmental events at greater ecological scales (Rayner 1997, Bartnicki-Garcia, Hergert & Gierz 1990). It is thought that DNA transcription followed by translation of particular sets of spliced RNA exons happens when oxidative and energetic conditions fail to become stabilised within the protoplasm after these first two levels of epigenetic response have failed to contain fluctuations of metabolic stress. In this way stress is filtered. If oxidative stress continues to cause metabolic dysfunction by altering the structures of carbohydrates, proteins and lipids above the capacity for membranes to maintain coherency via continual repair and replacement during so called "housekeeping" metabolism, signal proteins and metabolites may diffuse into the nucleus and begin a process of feedback there, leading to altered rates of transcription, transposition, de-methylation, ribosylation and glycosylation events. These result in a third-degree and genetic response consisting of up-down regulation and amplification and recombination of appropriate genes in response to environmental challenge (McClintock 1950, Gruntein 1992, Massey *et al.* 1999). For example, plants and fungi in particular, as well as many other organisms, possess multiple copies of iso-enzyme genes. These genes code for homologous enzymes which function in altered chemical contexts or temperatures due to slightly different amino acid sequences leading to conservation of quaternary folding despite vastly different conditions in which they present their active sites.

Finally if neither of the above three degrees of oxidative stress response can dampen or ameliorate ROI-induced damage inside hyphal protoplasm, non-linear unstable feedback may follow causing cell death via mitochondrial oxidative overload, membrane dysfunction, protoplasmic senescence and vacuolisation during apoptosis (Webber, Wakley & Pitt 1998). In so doing, superoxides and free radicals may proliferate through chain reactions, puncturing holes in the delicate membranous enfoldings of cellular ultrastructure by peroxidising membrane lipids (Oppenheimer & Stern 1939) and causing irreparable damage to regulatory

proteins, RNA transcripts and DNA. What is common to each of type of survival response, and also ultimate cell death, is that all emerge from underlying dynamic feedback processes which link the habitat environment at one scale to the context of inter-hyphal space and biochemistry of altered organelle and membrane molecules at the other.

How hyphae sense and respond to the changing heterogeneous conditions through which they grow and respond to the organisms they interact with (Tsunda & Thorn 1995) accounts for many developmental modes and shifts fungi perform in the forest (Olsson 1995, 1998). For example the transition from free-living yeast to hypha and the transition from separate hyphae to collective foraging chord are thought to be triggered by the same stimuli albeit at different scales. These oxidative stress-effecting stimuli, effecting development of hyphal boundary insulation, surface area to volume ratio and protoplasmic streaming through hyphal plumbing networks, (Eirich 1956) are aeration (desiccation), water availability and nutrient concentration (Rayner 1997). Developmental versatility arises through a process of feedback between changing boundary chemistry and redox power (Rayner, Beeching & Watkins 1995). **Decaying wood provides an excellent example of a changing chemical and physical context in which mycelium responds to change it has contributed to itself.** Feedback alters chemical and physical properties of wood, affecting the types of fungi that can persist within it over time.

In the past, views of wood decomposition have regarded interaction of fungi and wood simply as a linear process controlled by either insects or fungi. Wood has been viewed only as a static resource in which fungi operate. More recently, however, work has illustrated the complex interactions that can occur between fungal colonists and their plant contexts (Burden 1992) in the forest (Lengyel 1989, Frankland 1984, Frankland, Poskitt & Howard 1995, Coats, Rayner & Boddy 1995). Fungi that decay lignin have to break symmetry to adapt to constantly changing conditions (Lengyel 1989, Senior 1989) that are spatially, temporally, structurally and functionally heterogeneous (Ainsworth, Rayner & Boddy 1987, Sole & Bascompte 1993). This heterogeneity makes it extremely difficult to predict the exact outcome of fungal-wood combinations (Neubert 1997, Mercier 1982). Patterns develop from self-seeded processes of reciprocal feedback communicating between and affecting these systems over time (Dahlberg & Stenstrom 1991). It is necessary to see this starting premise as the un-constant context that frames any input insects impart to this already indeterminate, dynamic and heterogeneous system.

1.3 Developmental feedback in insects, comparison of insects and fungi

Fungus-insect interactions range from complete dependence of strict entomopathogenic fungi on insects as a food resource, to complete dependence of strict fungivorous insects on fungi as a food resource, accommodating a very wide spectrum in between (Guevara, Rayner & Reynolds 2000, Wilding *et al.* 1989). Only a handful of associations e.g. mushroom Drosophilids (Hanski 1989), and fungus gardens and macrotermites and *Termitomyces* fungi have received recurrent attention. By contrast a number of other trophic associations such as fungivory on fruit bodies or the assistance in cross-reproduction of phytopathogenic fungi by flies and beetles have received limited attention. Such interactions are expected to be particularly diverse in forests especially tropical forests where both insects and fungi display their greatest diversity (Hawksworth 1991, Pirozynski 1988). Among the antagonistic fungus-consumer relationships, the consumption of mushroom-like structures by insects has received considerable attention, but these studies

have concentrated on temperate Drosophilids, largely with a zoocentric emphasis, dealing with aspects of populations structure (Charlesworth & Shorrocks 1980, Worthen 1989 a, b), genetic diversity (Ashe 1984), competition (Grimaldi 1985, Grimaldi & Jaenike 1984, Shorrocks 1991, Shorrocks & Bingly 1994), predation (Worthen 1989 b, Worthen, Bloodworth & Hobbs 1995, Worthen & Moore 1991) and parasitism (Jaenike *et al.* 1992). Complimentary studies on the effects of fungi have been neglected due to the dominant viewpoint, which considers the fungal partner simply as the "resource". This position is analogous to that observed in the origins of plant-herbivore interactions where plants were only referred to as the "food source". Herbivory and fungivory can be seen as analogous relationships (Bruns 1984, Hanski 1989, Harper 1977) where trophic activities of consumers may have negative impacts on reproductive potentials of plants and fungi respectively.

How do ways in which fungi alter their phenotypes in response to changing circumstances compare with ways in which animals, in particular insects, develop? There are many parallels between development in the animal and fungal kingdoms. Principally these similarities relate to emergence of individual cell population specialisations from the sea of surrounding temporally-changing interactive information inputs which induce them (Foe 1989, Struhl 1981, Bryant 1970, Bart-Niki-Garcia 1990, Dahlberg & Stenlid 1990). For example, patterns made by neurones, trachea and hyphae are very similar in terms of cellular divergence, branching and networking so becoming part of larger-scaled anastomoses (Dahlberg & Stenlid 1995, Rayner 1997).

However, there are differences between insect and wood-decay fungal development, for example in the way in which insect development largely occurs within regions of tissue that become stably compartmentalised and fixed by physical or homeostatic signal distance-limits within which the developing insect larval and pupal cells diverge (Cooper 1998). Protective cuticular barriers in insects and fungi both consist principally of chitin. Thick chitin envelopes in insects provide a relatively constant and well insulated local signalling context around a group of dividing cells within the insect body plan (Foe 1989, Cohen & Honsley 1985). This is to be contrasted with indeterminate fungal and plant development where such compartmentalisation, when it does occur, may be both temporary and expendable. Hyphae therefore tend to be developmentally and genetically plastic (Cooke & Rayner 1984, Cooke 1970, Smith 1999, Zac & Rabatin 1997, Zac 1995, 1998) and thus resilient to disturbance.

So one of the initially subtle biological divergences which, through evolution, has produced many profound differences between organisms (Maynard-Smith 1998) has arguably been the presence, absence and permeability of a body envelope or boundary (Rayner 1997), especially to digest nutriment and receive energy from inside alimentary tracts, rather than be dependent on assimilating energy from the outside. To an insect, the impermeable chitin cuticle provides a safe chamber in which positions of cells relative to each other may remain fixed (Laurence 1975) in space and time allowing a repeated body plan (Slack 1988) to be returned to faithfully over many generations (Foe 1989, Gilmour 1965). Evidence for the importance of stability between cell positions (Laurence 1993, Osiewacz 1998) during insect development comes from the fact that if pupae are physically manhandled thereby changing cell positions relative to each other, the development process may be confused and often become fatal (Reynolds 1997).

The categorisation of indeterminate development for fungi and determinate development for insects are made even more blurred when re-examining larval death and re-assimilation during metamorphosis. Here, insects

pupate within protected chitin envelopes. Compartments and larval tissues then degenerate releasing the nutrients from which latent imaginal disc cells derive sustenance (Bryant & Levinson 1985) and seed the development of the adult insect's gut, muscles, eyes, brain, wings and genitalia (Lawrence 1993). Here, old larval cellular networks are broken down and re-assimilated into the new adult body plan via a process of re-cycling remarkably akin to that which fungi routinely use (Rayner 1997) to re-distribute themselves when new nutrients (figure 1.8) and water sources are encountered by the mycelium (Rayner 1998 b).

A perspective that succeeds in blurring the classic divide between fungal indeterminacy and insect determinacy is that of the foraging organism (Kroon & Hutchings 1994, Stephens & Krebs 1986). Time-lapse trajectories of animals form explorative behaviour patterns remarkably similar to those of roots, branches and hyphae (Franks 1996, Rayner & Franks 1987, Stephens & Krebs 1986, Brown 1999, Rayner 1997, Couzin 1999). Not only are trajectories similar but they are also developmentally flexible (Bainton *et al.* 2000, Barber 2000, Baker 1982) in a way which is perhaps universally appropriate to intimate, explorative, assimilative, high surface area to volume ratio existences within untempered fluctuations of non-linear heterogeneous environmental dynamics (Ritz & Crawford 1999, Rayner 2000, Barash 1978). When an organism or group of cells within a developing organism thin the insulation of protective envelopes they increase their sensitive surface area, and are brought more intimately into contact with other organisms, nutritional sources and abiotic components of their dynamic situation (Logan & Allen 1992, Lorenz 1985, Lotka 1956). It is at these scales, at which surface area to volume ratios can be most closely compared, that the indeterminacy of animals most closely reflects that of plants and fungi. These are scales at which life forms forage and explore for energy, water and nutrients and thus exhibit indeterminate patterns that are strikingly similar (Rayner 1997, Friese, Morris & Allen 1997, Stiller & Rossi 1998, Petraitis & Latham 1999, Lowman 1992, 1997). Such patterns may be produced by underlying chemical and physical processes that involve growth whilst maintaining cohesion (known in physics as "Expansion under contraction"), in other words context-channelled, location-specific reception of energy whilst minimising the resultant increase in entropy (Bascompte & Sole 1995, Rodenhouse, Sherry & Holmes 1997). As a consequence, the patterns produced by developmental feedback when a high surface area is required for foraging in a heterogeneous environment is likely to involve non-linear processes and possess highly fractal patterns with highly branched symmetry (Rayner 1997, Couzin 1999, Franks 1999, Gliek 1992, Falk, Bjornstead & Stenseth 1995, Meez 1981, Rodenshouse, Sherry & Holmes 1997). As such, the processes that create fractal patterns are more likely to cause symmetry breaking by entering non-linear states. Such states can be detected in long term temporal data sets using Lyapunov's exponent of asymptotic stability as an indicator of the state of deterministic ergodic or asymptotically stable chaos (Boukal & Krivan 1999, Ellner & Turchin 1995, Turchin 1992, Hastings, Hom, Ellner, Turchin & Godfrey 1993). We may thus predict that with increasing environmental heterogeneity, together with a mirrored branching of those fungal systems with a high surface area (assimilative) contact with these contexts, there will be displayed an increased likelihood of broken symmetry in development and behaviour and a reduced predictability over more insulated (and distributive) insect counterparts (Gould & Stinner 1984, Rayner 1997, Raven 1992, Pool 1989, Engen & Seather 1998).

There is substantial chemical, physical and mathematical biological evidence which suggests that whenever these patterns occur in nature they are not pre-determined from the onset by a particular pre-existing set of instructions. On the contrary it seems that initial conditions (Bascompte & Sole 1994) seed the potential (Geraci 1992) for indeterminate emergence (Badogan & Sutton 1994, Carlile 1987, Chapman & Leslie 1967)

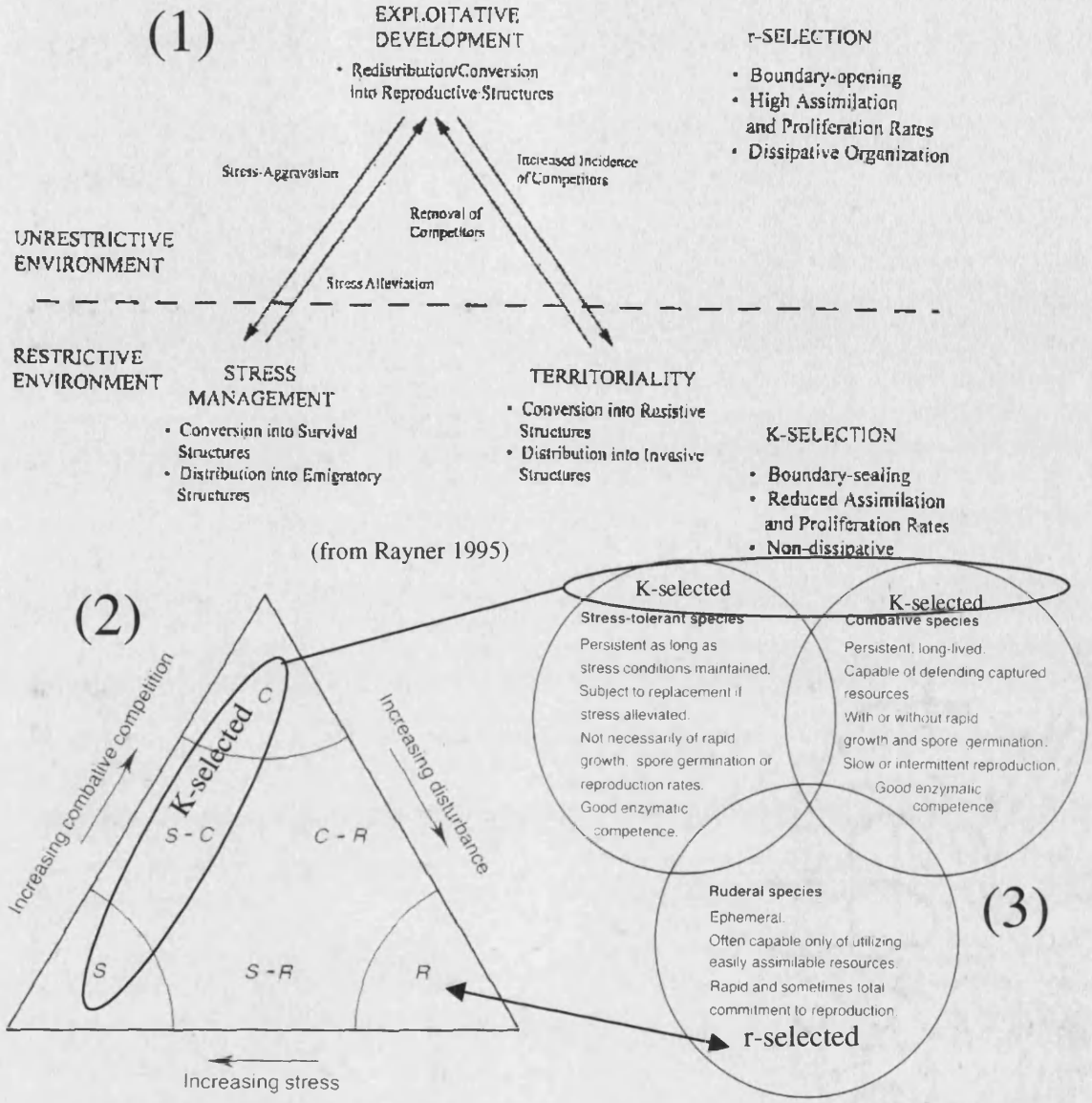
of symmetry-breaking (Kinler, Volgelstein & Lenganer 1999, Cabib, Duran & Bowers 1979, Alexandrov 1977, Alstad 1998, Arking *et al.* 1996) self-organised patterns (Emlen, Freeman & Graham 1993, Meinhardt 1982, Mervis 1999, Weissman 2000, Abrams 1999) through processes of associative and disassociative interplay (Lengyel 1989, Ruthen 1993, Glauert 1960) within complex environments (Tritton 1977, Rayner 1997, Couzin 1999, Thom 1967, Kauffman 1993, Hassel, Lawton & May 1976, Mopper 1998, Falck, Bjornstead & Opp 1998, Webb 1985).

Pattern generation in many complex non-living systems often involves physico-chemical convection and reaction-diffusion (Einstein 1926, Burger 1991, Anderson & Burras 1993, Acheson 1990) seeded by chemical and heat energy transfers across physico-chemical boundary gradients (Theraulaz & Bonabean 1995, Re 1992, Vernberg & Vernberg 1970) according to physical laws of thermodynamics (Berry 1998, Pelletiera 2000, Osserman 1986). Many of these same pattern-generating phenomena are also exhibited in living organisms (Ball 1999, Haken 1980, Boukal & Krivan 1999, Bressloff 2000, Brooks & Wiley 1986, Andrews 1995, Bagnold 1941).

There is a large subset of developmental changes that are therefore likely to be seeded by chemical and physical forces acting on and within biological membrane polarisations throughout and around cellular ultrastructures via reaction diffusion kinetics of populations of proteins and molecules of water and carbohydrate (Meez 1981, Bhatia & Szeso 1970, Thornton 1995, Ball 1999, Haken 1980). Through looking at both insect and fungal development in this way the scale at which a study takes place clearly becomes a major factor which can determine the extent to which one views the development of a species as being mechanistically deterministic. Deterministic mechanisms are absolutely controlled by a singular and simple non-cyclic linear event series in which entities are seen as atomistically separate and discrete (Vasiliauskas & Stenlid 1998, Fries *et al.* 1999). If scale is one of the key criteria which alters the way biologists view the determinability of events involved in development, then it is also worth asking whether other components of fungal ecology, such as the transition from ruderal early pioneer to K-selected late-stage colonist relative to scale of nutrient availability (i.e. distance to forage between nutrient molecules), are deterministically sensitive to perceptions of scale of view. Ecological relations to scale and stress levels for fungal development are illustrated in figure 1.7. Since it is possible to relate r-K-selection in insects to those of fungi with which they are associated, it might also be possible to bridge together separate disciplines of insect and fungal ecology with a common set of organisation principles. Furthermore, the bridging of insect and fungal ecology is perhaps most likely to be possible where co-existence is most evident, where the two taxa inhabit the same decomposing woody niches (Ghilarov 1964, Gibertson 1984, Whitlock 1994, 1995). It is hoped that this thesis will draw out these fungal-insect parallels and thereby strengthen the case to be made for inter-taxonomic developmental principles.

1.4 Insects, wood-decay and the case of paedogenic cecids

Both insects and fungi are vital in woody ecosystems (Begon *et al.* 1990, Strong *et al.* 1984, Rayner 1992.a, b, Roitberg 1992, Wilson 1992 a). Through the lengthy evolution of inter-taxonomic relationships, primarily between insects and plants, it is thought that insects and fungi have become co-dependent on woody plants and also co-existent via co-evolutionary interplays between all three taxa over evolutionary time (Taylor, Stubblefield 1990, Swift & Boddy 1984, 1987). Co-evolution between insects and fungi that inhabit dead



Schematic showing relationships between ecological disturbance, stress and combative stress (outside of triangle) on primary ecological strategy C=combative, S=stress tolerant, R = ruderal, and secondary strategies S-R= stress-tolerant ruderal, S-C= Stress-tolerant combative, C-R = Combative ruderal. (from Cooke & Rayner 1984)

(adapted from Cooke and Rayner 1984)

Figure 1.7 Schematics of ecological patterns and strategies exhibited by saprotrophic fungi. At top (1), an explanation of feedback in terms stress alleviation, nutrient status, boundary sealing mechanisms, ecological strategy and behaviour of an organism. Below (2) and (3) are two adapted schematics linking r-K selection to ecological behaviour, environmental conditions and pattern forming capability of living systems (adapted from Cooke and Rayner 1984).

wood has received little attention in comparison to that between living plants and fungi and between living plants and insects. It is likely that interactive states of fungi and insects are coupled as they relate to wood decomposition and the speed and spatial scale of the major ecological cycles of carbon, phosphate and nitrogen (Swift & Boddy 1984, Hatcher 1997). Such interactive roles could be induced by initial chemical and physical processes leading to wood-decay, and inducers of consequent changes in chemical and physical processes during decay towards humus and soil (Swift & Boddy 1970, 1984, Cooke 1984). Processes apart from wood-decay that are thought to be effected by insect-fungal co-evolution are pollination, seed dispersal and fruit ripening (Hawksworth & Pirozynski 1998, Pirozynski & Malloch 1988, Futuyma & Statkin 1983). The loosening and detachment of bark on the surface of decaying logs has not, as far as I know, received any attention from this co-evolutionary perspective before. Co-evolutionary trends between trees, insects and fungi could shed light on the evolution and propensity for symbiosis. It seems that symbiosis may be regarded as a coupling mechanism over biological history that mirrors co-evolution and co-existence (Tokeshi 1999). Changes in boundary shape and properties over time influence the relationship between inner (contained) contexts and outer environmental contexts (Rayner *et al.* 1999, Forman 1995, Morin 1999, Mouquet & Loreau 1999). An outer context may consist of an array of biotic and abiotic factors, the proportions of which may vary as greatly as inner contexts. It may be that environmental-flux induces feedback-effects on the permeability of boundary conditions that influence exchange rates and directions of ROI, water, and nutrients between partners (Forman 1995). Thus the type of one organism's symbiosis with another can be greatly altered by the fact that boundary properties are dynamic and sensitive (just as boundaries are in a constant state of flux, so too are symbiotic relationships) (Margulis & Sagan 1995, Magulis 1998, Rayner 1997). This is of great importance in ecology (Margulis 1998, Goodwin 1994, Ho 1993). More so in understanding the way in which complex life systems have evolved from simple pre-biotic chemical and physical relations (Kauffman 1993, Ho 1993, Rayner 1995, Schwemler 1989). The role of boundary semi-permeability in allowing flow-through and block of certain factors between partitioned contexts, according to the size of holes within the boundary, allows the evolutionary significance of early lipid micelles to be appreciated more fully (Baskin & Norde 2000). The origins of organic (as in biological) order are perhaps likely to be due to the formation, movement, enfolding and morphogenesis of dynamic lipid boundaries (Thom 1992), moved by fluxes of chemical and physical potentials but tempered and constrained by degrees of flexibility and permeability to restrict such feedback only to those morphogenetic adaptations which increase survival.

As with lichen formation, biofilm build-up and the endosymbiont theory, perhaps when there are a multitude of boundary surfaces which maintain coherence despite partial mutual enclosure, as in most symbiotic and co-evolutionary developments (Margulis & Sagan 1995, Goodwin 1998, Schwemler 1989, Rayner 1997), a process of boundary proliferation occurs which increases the overall heterogeneity and structural diversity of the system (Rayner 2000).

This study addresses the way populations of fungi and insects co-exist symbiotically within decaying wood in an attempt to begin to ask similar questions about the relationship between insects and fungi that have previously been asked about insects and plants (Guevara 1998, Bakker, Blair & Knapp 1999, Hatcher 1995, Worthen 1989 a, Worthan, Mayrose & Wilson 1994). One of the reasons why fungal–insect co-evolution (Hochulli 1996) and symbioses (Hodkinson & Huges 1996) may have been under-reported (Ahmadiain & Paracer 1986) is that fungi live, for the most part, hidden within dead or living parts of plants (Creffield

1996, Tokeshi 1999, Hickin 1975, Howe & Westley 1988, Hunter 1999, Hoevemayer 1999). Indeed, architecture of soil and plants (Tokeshi 1999, Torgerson & Bull 1995), which provides most cavity insect accommodation, could perhaps be induced by fungi. However, literature regarding insects within wood rarely appreciates the importance of latent or active fungal phenomena with which the insects have certainly been co-existing (Beavon 1987, Hickin 1975). So this study will investigate how decaying plant matter may be shaped by an interplay between fungal and insect life-cycles (Ingram 1992), ecological successions, and possible developmental feedback effects (Lacey 1984, Moore 1996, Martin 1999, Klironomos & Kendrick 1995, Larson & Jakobson 1996). Recently research has been carried out in tripartite interactions between interactive fungi, plants and insects (Hatcher 1995, Hatcher & Ayres 1997, Hatcher 1997) which suggests that negative, constitutive or synergistic physical and chemical metabolic effects may be occurring. Here synergistic is used to describe the effect of combined attack of insect and fungus on living plants. The effect of insect and fungal treatments on plants can be described as synergistic when they are greater than the effects of fungi and insects when added in isolation. Later on in this thesis, synergistic effects describe the formation of chemical reactives when two or more metabolic systems interact such that the response is more than the addition of components from the same chemical systems held in isolation. Haken, who worked on theoretical synergetic states in biological and membranous systems, distinguished chemical synergistic effects from physical synergetic effects. Haken defined effects as synergetic when a combination of forces produce morphogenic or topological outputs which are completely novel and not expected from the sum of initial conditions which seed them (Haken 1980). Simple examples of such effects would be sudden cavitation of water under pressure from a propeller, or the breaking into a turbulent flow when two non-turbulent flows coalesce (Haken 1980, Favre *et al.* 1988). Haken suggests on the basis of mathematical models that it may be possible that initial synergistic (chemical) processes may seed synergetic (physical) forces with their potential to produce shape and structure in biological systems and assemblages (Haken 1980).

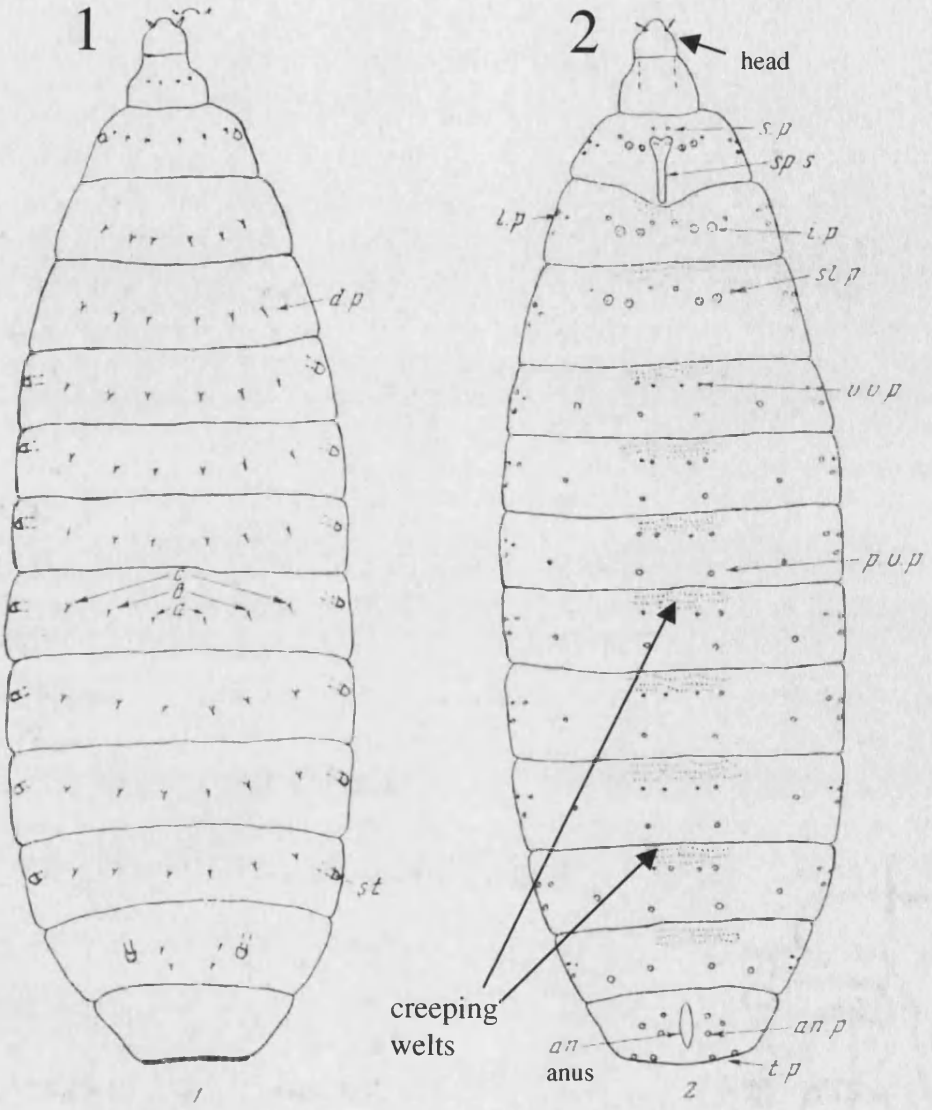
It has been shown that plants defend themselves against combined effects of insects and fungal complexes (Klepzig, Smalley & Raffa 1996). Mycetophilid fungus gnats of genus *Bradysia sp.* have been shown to be attracted to inter-specific somatic incompatibility boundary zones such as those produced in wood between wood-decaying fungi (Boddy, Coats & Rayner 1983). The adaptive significance of this observation has not been investigated (Swift & Boddy 1984). This thesis will investigate how synergistic and synergetic effects of interactivity between taxa may be relevant to the many orders and families of insect which inhabit dead wood (Hakin 1975, Cooke & Whipps 1987, Dajoz 2000).

A good candidate for empirical study in this respect is a sub-group of the dipteran fly family Cecidomyiidae (Grover 1988). The name Cecidomyiidae comes from Greek "kekid" meaning gall and "myiidae" – meaning fly (Chambers 1983). This large family of very small flies is well known for forming galls on living plants (Kant & Ramani 1998, Mamaev & Krivosheina 1993). The fact that many of these galls, within living tissues of plants, become full of hyphae has raised little research interest on the possible insect-fungal relations therein (McLean 1994, Batra & Lichtwardt 1963). Recently a study showed a black-pigmented yeast to be symbiotic with a gall-forming midge (Herman, Hyanm, Hexander 1993). Other insect galls, such as ambrosia galls, though not formed by the family Cecidomyiidae, remain a classic example of insect fungal mutual symbiosis (Batra & Lichtwardt 1963, Bisset & Barkent 1988, Cooke & Rayner 1984). Aside from the hormonal and metabolic exchanges through which living plant development is topologically shifted to

develop gall cavities in which insect larvae grow (Toda, Kimura & Tuo 1999, Redfern 1997), there lies an intriguing possibility that such cavity-forming organisms may shed light on development of boundary configurations and associative morphogenies via their interactive metabolism. This thesis investigates such tripartite co-existent systems between tree, insect and fungal community vis a vis wood decomposition, bark detachment and wood cavitation processes.

A subset of some of the smallest sized sub-families of Cecidomyiidae dipteran fly family live exclusively in dead and decomposing substrates often in the sub-cortical zone between bark and underlying sapwood. They exist as obligate and specialised mycophagous larvae with very reduced adult phases (Darwin 1859). Indeed several genera have evolved to possess no adult phase at all (Mamaev & Krivosheina 1993) and possess a high female sexual skew in their aggrarious populations. From now on, the use of the word "cecid" in this work refers to this particular sub-group of sub-cortical, dead wood and fungal dwelling members of the Cecidomyiidae family. These particular cecids are common but often mistaken as "midges" or "gnats" (Sciaridae) since they are so small. Most species only reach 2-3 mm in length, have whorled and long curvy antennae, fine hairs around their wings and lack mouthparts. In addition, a few cecid genera only reproduce asexually without leaving the larval stage in a most extreme example of the results of selection towards a reduced life-cycle (Darwin 1859, Kahle 1908, Gabritschevsky 1928, Springer 1915). This form of reproduction, termed "**Paedogenesis**", was first reported in 1862 by Wagner from cultures he made of *Miastor metraloas* larvae which he had discovered under the bark of rotting beech, lime, elm, ash and rowan stumps in Russia (Darwin 1859, Wyatt 1961, Wagner 1863, Kahle 1908, Camenzind 1963, Cuellar 1977). The paedogenic reproduction cycle, a form of alternation of generation that fascinated Darwin, is a form of asexual female parthenogenesis with a difference (Wyatt 1963, Cognetti 1965). All young are born together in one maternal-fatal semelparous birth in which the mother larval cuticle is ruptured releasing numerous young daughter larvae (Wyatt 1961, Ulrich 1934, 1943, 1962). Cecids possess a very high genetic polyploidy of nearly 40(n) which, via a process of complex cytogenics (White 1946) analogous to some plant polyploidous systems (McClintock1950, 1953, 1961, 1983), enables chromosome mitotic copies to be reassorted and selected according to the environment, with the rest carried but masked through a mechanism of chromosomal duplication and "elimination" (Went 1977, Ulrich, Petalas & Camenzind 1972). Female daughters grow fast, with few or no moults, and follicles begin to develop within their ovaries without fertilisation (Camenzind 1982, Went 1977). Very occasional male larvae are produced in particular and stressful conditions that are very hard to replicate in laboratory culture (Went & Camenzind 1977). Males pupate at the same time as females by breaking out of paedogenic reproduction under stressful environmental stimuli, and may mate (Camenzind 1963). Females may land on new mycelium and lay up to four eggs (Camenzind 1963). Only under certain conditions (Went 1975) do eggs develop young, again this is extremely hard to replicate in laboratory cultures (Camenzind 1963). Eggs develop into paedogenically-reproducing females (Went & Camenzind 1977, Nikolai 1958, 1961). If conditions are favourable, paedogenic cycles of larval mother to daughter reproduction can occur indefinitely (figure 1.9), prompting the question why males and why sex at all (Went 1979, Dagg 2000)?

Cecids exist for the major phase of their life-cycles as obligate mycophagous larvae inhabiting mycelia. It has been shown that cecid larvae are resistant to insecticides suggesting that they can handle the ROI-induced oxidative stresses of fungal-caused lignin catabolism (White 1977). Figure 1.8 shows generalised cecid larval cuticles. On the ventral surface, anterior in each segment, are rows of serrated projections called



1 – dorsal view; 2 – ventral view; an. – anal opening; an.p. – anal papillae; d.p. – dorsal papillae (a – central pair; b – intermediate pair; c – marginal pair); i.p. – intercalary papillae; l.p. – lateral papillae; p.v.p. – posterior ventral papillae; s.p. – sternal papillae; sl.p. – sublateral papillae; sp.s. – spatula; st. – stigma; t.p. – terminal papillae; v.v.p. – anterior ventral papillae

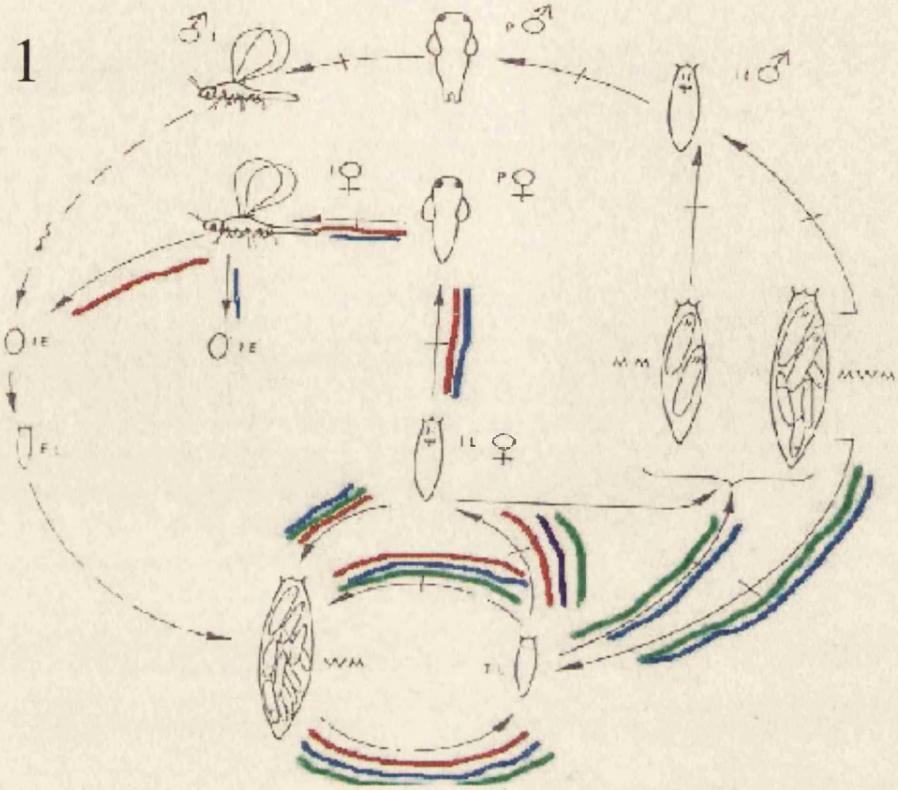
Note from the editor: The terminology of larval papillae used in this volume slightly differs from the terminology used by most specialists (e.g. Möhn 1955b) in the following way: i.p., intercalary papillae – lateral papilae; l.p., lateral papillae – pleural papillae; sl.p., sublateral papillae – inner pleural papillae

Figure 1. 8 Generalised cecid larval body plan. The features illustrated here show great variation between genera and species. When considered together they provide a key to identification (from Wyatt 1967). 1 shows a dorsal and 2 shows a ventral view.

creeping welts, to aid locomotion, and the rest of the cuticle surface is covered at regular intervals in sensoria. There is a tiny head in proportion to the rest of the body size, which has a tiny set of mouthparts. These mouthparts are small enough to snag and rupture individual hyphae (1μm wide). These cecids feed by clasping, piercing and ripping hyphae and allowing protoplasmic streaming to spill hyphal protoplasm around them (Springer 1915) which they suck into the oral cavity.

Most of what is known about cecid ecology is by association between cecids and different tree-bark species in the field (Mamaev 1993, Buxton 1954, Graves 1985, Batra 1963, Fedotova 1995), by which new cecid species have been documented, or by producing conditions in agriculture where cecids become pests, most notably in intensive cultivation of *Agaricus bisporus* (Wyatt 1964, Wyatt 1959 Hussey & Wyatt 1958, 1957, 1961, 1962). This study aims to investigate an aspect of cecid ecology by linking cecid activity with the macro-ecological process of fungal wood decomposition and the attraction of cecids to boundaries between wood-decay fungi. There are many cecid species that live in association with decaying wood, under bark or within rotting sub-cortical tissues. It is most unlikely that all species resident in the UK have been discovered (Wyatt 2000). Ecological records are rare despite the fact that such wood-inhabiting cecid larvae are some of the commonest forest insects (Wyatt 2000). These include two Cecidomyiidae sub-families with which this thesis is concerned, and about which results are interpreted as a case study. Firstly is sub-family Heteropezini, which includes *Miastor metraloas*, *Heteropeza pygmaea*, *Brittenia fraxinicola* and a long intra xylem-vessel dweller called *Leptosyna nervosa*. Sub-family Lestremiinae, which includes *Mycophila speyeri* (Wyatt 1967), is the second. The feeding specialisation of larvae is designated mycetophagy (Mamaev & Krivoscheina 1993). Cecid ecology is primarily the study of paedogenic larvae since the duration of larvae in comparison to adults is overwhelming (Hunt 1996, Gabritschevsky 1928, Mamaev & Krivoscheina 1993). My work has thus principally concerned the ecology of dipteran larvae that inhabit decomposing wood. It has been found that larvae of *Miastor metraloas* perish in absence of fungi under bark (Mamaev & Krivoscheina 1993). The life-cycle options for 3 species of paedogenetic cecid larvae studied in this project are shown in figure 1.9. **Adult female *Brittenia fraxinicola*** [Edwards] **were first discovered in 1941 in Whitby, Yorkshire on ash trunks**. The only other record was made by Wyatt in 1958 in Clapham woods, Sussex. Wyatt discovered larvae of a potentially new species under bark of dead hazel, ash and oak. He cultured them on the fungus *Stereum hirsutum* and found that they reproduced paedogenetically. On two occasions pupae were produced, which both died but one formed the identifiable adult features of *B. fraxinicola* (Wyatt 1967, 2000). Small daughter larvae (born 1.4 mm long) take about two weeks to go through two instars to become larger mother larval hemi-pupae (between 2 and 4.7 mm long) before discharging between six to 12 young (Wyatt 1967). **This study will focus on *Brittenia fraxinicola* as the main case study, although experiments were also performed on *Heteropeza pygmea* and *Mycophyla speyeri***, and will ask distal (evolutionary) and proximal (ecological) questions which relate paedogenic features such as large polyploidy, loss of sexual reproduction and simplification of life-cycle to the specific conditions of the sub-cortical zone and developmental feedback processes involving fungi in whose mycelia these larvae dwell. This is the first study, as far as I know, which considers the natural ecology of cecids in decaying wood, whilst previous studies have concentrated on taxonomy and analysis of life-cycles and their polyploidal chromosomal silencing mechanisms (Ulrich, Petalas & Camanzind 1972, Ulrich 1934, 1943, 1963, Went 1973, 1975, 1979, Fux 1974, Gabritschevsky 1928, Went & Camanzind 1977). **This work also represents the first ever study of the ecology of *B. fraxinicola* and the third study of its general biology since the species was discovered** (Wyatt 1967 & Wyatt 2000).

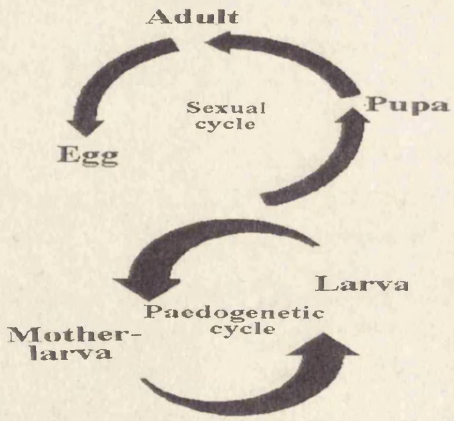
1



Red = *Mycophila speyeri*
Blue = *Heteropeza pygmaea*
Green = *Brittenia fraxinicola* (field species)

Life Cycle of *Mycophila speyeri* (Cecidomyiidae, Diptera)

2



Brittenia fraxinicola feeding on *V. commedens* fungal mycelium.

3

Figure 1.9 (1) shows a generalised life cycle of peadogenic cecids on to which have been superimposed those parts present in 3 particular species; *Mycophila speyeri* (red), *Heteropeza pygmaea* (blue) and *Brittenia fraxinocola* (green) – the field species upon which much of my studies have been focussed. (2) is a simplified version of the life cycle of *Mycophila speyeri* indicating where the paedogenetic phase of the life cycle occurs. (3) shows *Brittenia fraxinicola* larva feeding in basidiomycete mycelium of *Vuilleminia commedens*.
(1) - adapted from Mamaev and Krivosheina (1993), (2) - courtesy of Justine Hunt (Hunt 1996).

This is also the first study to simultaneously investigate the combined interrelationships between insect larval and mycelial components within decaying wood from the perspective of both insect and fungus development and effects on bark detachment and wood decomposition. Other work has been carried out from the perspective of insects and wood alone, for example regarding mycophagous larvae of death watch beetles *Xestobium rufovillosum* in structural timber (Campbell & Bryant 1940, Fisher 1937, 1938, 1940, Fisher & Parkin 1930), and the density of wood (Fisher 1941), but not on fungal decay communities (Guevara 1999).

1.5 Influence of insect activity on fungal communities

Many insect species have been shown to be either obligate or facultatively mycophagous. As such, insects are the most biodiverse of the many other animal groups that eat mycelium and fungal fruiting bodies (Abbot, Berecome & Rayner 1972), disperse fungal spores (Ingold 1951, 1971, Anderson, Rayner & Walton 1984) and live inside the mycelial-filled domains of living and decomposing plants (Hatcher 1995, Swift & Boddy 1984, Dowding 1984). Few attempts have been made to compare the relationships mycophagous insects have with fungi to those that herbivorous insects have with their host plants (Bultman 1995, Geuvara & Dirzo 1999). Most studies have restricted themselves to regarding the fruiting body of the fungus as the only part of the organism worthy of attention. Few have looked at interactions between mycelial growth and larval development, or at the combined effects of insects and fungi on decomposition and nutrient cycling processes (Swift & Boddy 1984).

The influence of insect activity on fungal decay communities has been demonstrated in studies of Attine leaf-cutter ants involving a reciprocal exchange of metabolic communication signals (Cooke 1977, Huxley 1949, Pirozynski & Hawksworth 1988). This influence has enabled insects to become attuned to fungal perceptions of oxidative stress (Cooke & Rayner 1984, Rayner & Todd 1979). Also symbiotic roles of fungi on the activity of phloem-feeding scale-insects have shown fungi to have evolved to biotrophically invade a small proportion of scale-insect phloem feeders, to obtain sugars, and in return the fungus cloaks the whole scale-insect colony with an insulated mycelium (Cooke & Rayner 1984) which protects insects from abiotic factor fluctuations as well as predation. However, even today, a real lack of entomo-mycological interdiciplinarity exists, except regarding those entomopathogenic fungi that are obligatory dependent on using insects as their food source where some recent work has been carried out (Charnley 1997, Clarkson & Charnley 1996). Currently many entomologists believe that insects are fundamentally causative in the decay of wood (Hickin 1975, Tokeshi 1999, Wilding *et al.* 1989) whereas mycologists believe that many of these insects could not even digest the cellulose and lignin without **symbiotic** and highly **co-evolved fungi** which live in their alimentary tracts (Pirozynski & Hawksworth 1988, Mishra 1985, 1989, 1991, Martin 1984, Swift & Boddy 1984) and that lignin is actually broken down by **fungal biochemistry** *per se.* (see Appendix 2 regarding habitat of violet click beetle *Limoniscus violaceous*). Wherever the truth lies, it seems highly likely that there has been a high degree of **evolutionary and ecological interplay between dead-wood-inhabiting insects and fungi** over the course of geological time (Cooke & Rayner 1984) which mirrors the interplay between fungi and plants (Crawley 1983) and between insects and plants. So here then is the topic to be addressed by this thesis: **a case study using cecid larvae on the properties and effects of insect-fungal co-existence on wood-decay and *vice versa*.**

The effects of **fungi on insects and of insects on fungi** are likely to be of **indirect effect** (Fryar 1998, Hanski 1999, 1989, Bonsall & Hassel 1999, Gange 1999), and **direct effect** (Hatcher 1997, Anderson & Ineson 1984, Ashe 1984, Baltham & Mathews 1996) and may demonstrate synergy of chemical and physical processes (Hatcher & Ayres 1997). Dynamic changes that have been shown to take place amongst types of ecological interplay between metapopulations of heterogeneous patch size (Anderson 1987), scale, nutrient quality and within population dynamics (Diffendorder 1998, Dighton & Mason 1985, Anderson & Kohen 1998, Harison & Hastings 1996) are other aspects to be considered. It has been suggested by Tokeshi that **heterogeneity increases biodiversity** and *vice versa* (Tokeshi 1999) in a macro feedback loop. This is relevant for reasons already introduced regarding developmental indeterminacy. This project will also investigate heterogeneity through **fractal geometry** of the ecosystem in question: a hazel coppice, using a way of defining heterogeneity as the amount of boundary proliferation and efficiency of space filling which occurs (Ramsdale & Rayner 1997). Much population dynamics relates to **feedback-induced** change at underlying chemical and physical scales (Dahlberg & Stenstrom 1991). Many types of ecological and metabolic cycle are interrelated, and coupled, by the process of feedback. **Our existing understanding of the role of insects does not allow for feedback between insects and fungi. Neither does our present view allow for an influence of the process of wood-decay on dead wood structures as accommodation potential for particular insect larvae** (Heino 1998, Holt 1984, Rayner & Webber 1984).

A preliminary investigation of the habitat of the rare and endangered violet click beetle *Limoniscous violaceous* in 1996 showed it to inhabit large, mature hollow oaks (*Quercus robor*) with very extensive pseudosclerotial plate (PSP)"curtains" hanging down inside the hollow making a brittle black structure analogous to honeycomb of very great surface area. It has been demonstrated that this **pseudosclerotial plate (PSP)** forms between fungi of different species as a response to oxidative reactions, which produce hydrophobic phenolic and turpenoid structures. It is thought that these chemicals then become sequestered and melanised into a particular complex architecture through **synergistic interaction**. The preliminary investigation of *L. violaceous* suggested that PSP was a major feature of the **development** of the **hollow decayed heartwood's architecture**. A report from this investigation is shown as Appendix 2. It is likely that the violet click beetle is rare because of the exclusivity of this particular architecture of pseudosclerotial plate. This emergent architecture represents a durable legacy produced from the past metabolic synergy from an interactive fungal community living in mature oak of substantial girth found only where these large and ancient trees remain. The *L. violaceous* interaction with the fungal decay community of oak was thought to be at the climax end of succession decomposition of heartwood in these large trees. An extension of this logic, by comparison, is made at smaller scales with regard to the more **ruderal**, pioneer fungal and **insect communities** that **inhabit dead hazel** poles of coppiced woodland near Bath. So this empirical case study of **Cecidomyiidae-Basidomycete-Ascomycete-PSP** formation is investigated with reference to other successions of decomposer fungi and associated insect fauna towards the kinds of situations exhibited with other Diptera as well as larger saproxylic beetles such as *L. violaceous*.

This is the first project, as far as I can tell that aims to explore links between processes by which insects interact with patterns of fungal decay of wood, to PSP patterns and cavity architectures. **Effects of insects on mycelial community development have not been investigated before within dead wood.** Where roles of insects have been considered, they have been restricted to considering insects as transport

vectors for fungal spores or protoplasts, or as destroyers of fruiting bodies. Such studies have overlooked the effect of insects on mycelium (Swift & Boddy 1994, Morin 1999, Speight, Hunter & Watt 1999). Previous approaches to insect and fungal co-existence within wood decomposition processes do not allow for reciprocity of feedback effects (Blaney 1999, Buxton 1960, Courtney, Kibota & Singleton 1990, Bruns 1984, Swift & Boddy 1984). Reciprocal effects have been reviewed by Hatcher, concentrating on spatially indirect interactions between living plants, pathogenic fungi and insects (Hatcher & Ayres 1997). As Hatcher points out, usually previous studies have only considered one type of effect and in one direction only: fungi on insects or fungi on plants. Little understanding of the nature of tripartite or more complex interactions can be drawn from the basis of such duplexes (Hatcher 1997, Hatcher & Ayres 1997, Morin 1999, Tokeshi 1999, Maurer 1999). Classic evolutionary theory considers adaptation of an organism to its surroundings but not the changes brought about to the surroundings by the organism's repeated life-cycles (Depew & Weber 1997, Bey-Bienko 1964, Bever, Westover & Antonovics 1997). The concept that the two dynamic patterns that describe fungal communities and **cavity architecture in dead wood** might be mutually and **reciprocally enhanced by insect co-habitants** has not aroused the curiosity for empirical investigation before now.

1.6 Aims of study

The aim is to investigate co-existent interaction effects between developing distribution patterns of insects and fungi during wood decomposition. Such reciprocal developmental interactivity between insect and fungal inhabitants of decomposing wood is examined at field-population and metabolic scales. This project also seeks to break new ground in the study of **inter-specific interactions between saprotrophic fungi of different sub-divisions**. Other aims are to investigate the nature of insect and fungal distribution within sub-cortical bark layers of decaying hazel (*Corylus avellana*) and the surrounding habitat and identify the best set of scales with which to proceed with laboratory experiments regarding the chemical and physical potentials for emergence of organism distributions, and to relate tripartite relations of dead wood, fungal and insect communities with macro-ecological processes such as decomposition of woody debris, resource partitioning and symbiosis.

To achieve these aims the study uses obligatory mycetophagous cecids as a case study to investigate interrelationships between plant, insect and fungal members of three kingdoms. **In particular the project aims to relate cecid-wood-fungal PSP understanding to the process of lignin decomposition, heterogeneity, ecological assemblage, metabolic coupling, surface area and symbiosis**. This case study evidence will be used to draw parallels where possible to suggest ways of increasing our understanding of fungus-insect relationships in woody plants and to explore conceptual links between heterogeneity, co-existence, adaptation, symbiosis, formation of soil, contraction of nutrient cycles and **biodiversity conservation**.

The case study of this cecid-wood-fungal-PSP system also has bearing on reciprocal dynamics involved in the patterns and processes of separate fungal mycelia, insects and also combined insect-fungal systems (Allen 1991, Adams, Williams & Todd 1984, Hanson & Hamelin 1999). Thus this work is of relevance to other work carried out on insect and fungal dispersal, metapopulations, source-sink dynamics, ephemeral habitat patchiness and general co-existence theories (Tokeshi 1999, Morin 1999, Maurer 1999, Speight, Hunter and Wyatt 1999). Relating the development of foraging trajectories to the boundary configurations

between fungal decay columns and cavity-sizes (Lussenhop 1992, Zhou *et al.* 1997) may also help understanding of *Ophiostoma ulmi*, the Dutch elm fungus, and its Scolitid beetle vector, development of site-specific foraging and brood chambers under bark (Swift & Boddy 1984, Webber & Brasier 1984). Such environmental channelling of foraging patterns may help to explain how life-cycles of insects have evolved to co-habit with fungi and adapt to the indeterminate ways of their dynamic hosts (Swift & Boddy 1984, Webber & Brasier 1984, Cooke & Rayner 1984, Rayner 1997). This project also aims to investigate how surrounding abiotic and biotic conditions influence interacting insect and fungal partners and the emergent patterns they produce in combination. A further aim is to integrate evidence into what has previously been demonstrated regarding cecid genetics, to discuss how conceptually the environment may shape evolutionary processes at the genetic level in natural heterogeneous ecosystems.

A multiple-scaled approach is used which combines several scales of field work with several scales of laboratory work in which pure and interactive combination fungal cultures with and without treatments of cecid larvae were grown first on agar and subsequently in wood. This approach enables reciprocal effects to be detected from population to **enzyme and antioxidant levels**. The study seeks to experimentally determine what role cecids play in altering fungal community composition within dead hazel wood, and what role fungal community dynamics plays in altering development of distribution patterns of co-habiting cecid larval life-cycles and foraging trajectories. The hope is to use cecids and fungi which decay hazel (*Corylus avellana*) as a case study in reciprocal developmental feedback and pattern emergence. In particular the investigation seeks to find out if data agrees with theory on mutual, combined, constitutive or synergistic effects on patterns of boundary development. Finally the effects of both taxa are considered on the development of a succession of initial to emergent cavity-spaces in the sub-cortical matrix of triple-layered bark of dead hazel wood.

1.7 Field and laboratory studies, the urgency of new methods and synthesis

Above all, ecology is the science of the natural environment; an environment in which beings interact, adapt and survive the challenges posed by the living and non-living components of their milieu. Somehow the combined efforts of countless species, including their coupled living processes (Lovejoy 2000, Levin 1999), create equilibrium or chaotically stable emergent forms when their interaction patterns coalesce (Whitehead 1978, Ball 1999, Gleick & Porter 1996, Watts 1999, Bertalanffy 1968, Rayner 1997). These coalescent shapes emerge as changeable boundaries and surfaces, presenting themselves as outwardly recognised living structures of the world's ecosystems and biomes (Rayner 1997). However, just as ecologists are getting closer to explaining the hidden processes which seed such complex and diverse ecological networks, we see our study material destroyed and converted in an accelerating global economic and philosophical requirement to see the material existence of our species de-linked from cycles (Capra 1996) and developed along commercial, technologically-sophisticated, far-removed and dis-localised patterns (Lovejoy 2000). This anti-biotic conversion (Rayner 2000) has resulted in a process of systematic disruption and delay of natural cycles of microbial decomposition and plant re-forestation, thereby massively expanding the spatial scale and hindering the temporal speed of vital water, carbon, nitrogen and phosphorus cycles. **Without the unique biology of countless species of fungi and bacteria, these fundamental ecological cycles could not exist. Furthermore, the pattern of cycle-expansion and cycle-delay, caused**

by the inter-connected processes of dis-localisation, de-forestation, habitat-fragmentation, soil-erosion, water-depletion and climate-change (Lovejoy 2000, Levin 1999), has already precipitated the sixth mass, and most rapid extinction-event of life's geological history, and will probably cause our own extinction unless we embrace sustainable change (Wilson 1992 a). In order to curtail the present rates of destruction to these complex and fragile natural cycles (Levin 1999, Wilson 1992 a), it is therefore of great importance, as ecologists, mycologists, entomologists, micro- and molecular-biologists, that we learn to communicate actually what is so special about living systems and their environments (Wilson 1992, Tarnas 1991, Gleick & Porter 1996). To achieve this we need both to match our understanding with empirical data, and also to increase public perception about what living systems actually comprise (Whitehead 1978, Wilson 1992 a, Levin 1999, Rayner 1997, 2000, Capra 1996, Ho 1993). This could enrich a philosophical, cultural and economic value of living as opposed to artificial processes (Wilson 1992 a), i.e. how our civilisation relates to life, so reducing life's destruction (Russel 1914, Goldsmith 1992 Whitehead 1978, Collingwood 1960).

Considering our limited knowledge of biodiversity and relations between species, and the speed with which habitat destruction is proceeding globally, there is a desperate need for inventory work so we may at least know which populations could be destroyed if, for example, a dam were to be built or a forest cleared. **The methods employed for biodiversity inventories vary considerably, but have yet to be standardised for fungi. As a result, most environmental impact assessments overlook fungi and other microbes.** A standard rapid fungal biodiversity assessment protocol is needed to compliment those that already exist for birds, mammals, amphibians, insects, trees and ground vegetation. With regard to the fungal kingdom there is an urgent need for rapid biodiversity assessment techniques to be developed which also meet the balance between detail and breadth of knowledge gained (Hawksworth 1993, Cannon 1998, Starck & Davies 1996, Hyde 1997). Moreover, it may be necessary to accept some compromise between depth and breadth in protocols that could be applied also by non-mycologists or school children conducting biodiversity surveys whenever they can.

The methods and organisms chosen to answer ecological questions can be curtailed by the available time for study and also by the distance between laboratory facilities and habitat in question, as well as costs of equipment and travel. As suggested by E.O Wilson, the biodiversity of life is in desperate need of scientific investigation because what we understand better we are less likely to destroy (Wilson 1992). **The idea of this project is to integrate modes of laboratory, metabolic-scale and field-population-scale investigations in order to understand the processes which give rise to a diversity of interactive distribution patterns of species which inhabit the sub-cortical zone of decomposing branches and trees.**

Fieldwork is the only time when biologists get to observe how their study subjects actually live, behave and distribute their populations in the ecosystems of which they are a part. Time spent in the forest continually enriches ecology with new insights, ideas and possibilities, and provides the initial questions for experimentation. Given the diversity of un-studied life in forest and reef ecosystems (Wilson 1992 a), basic field observations must surely long continue to be the genuine source of our scientific inspiration.

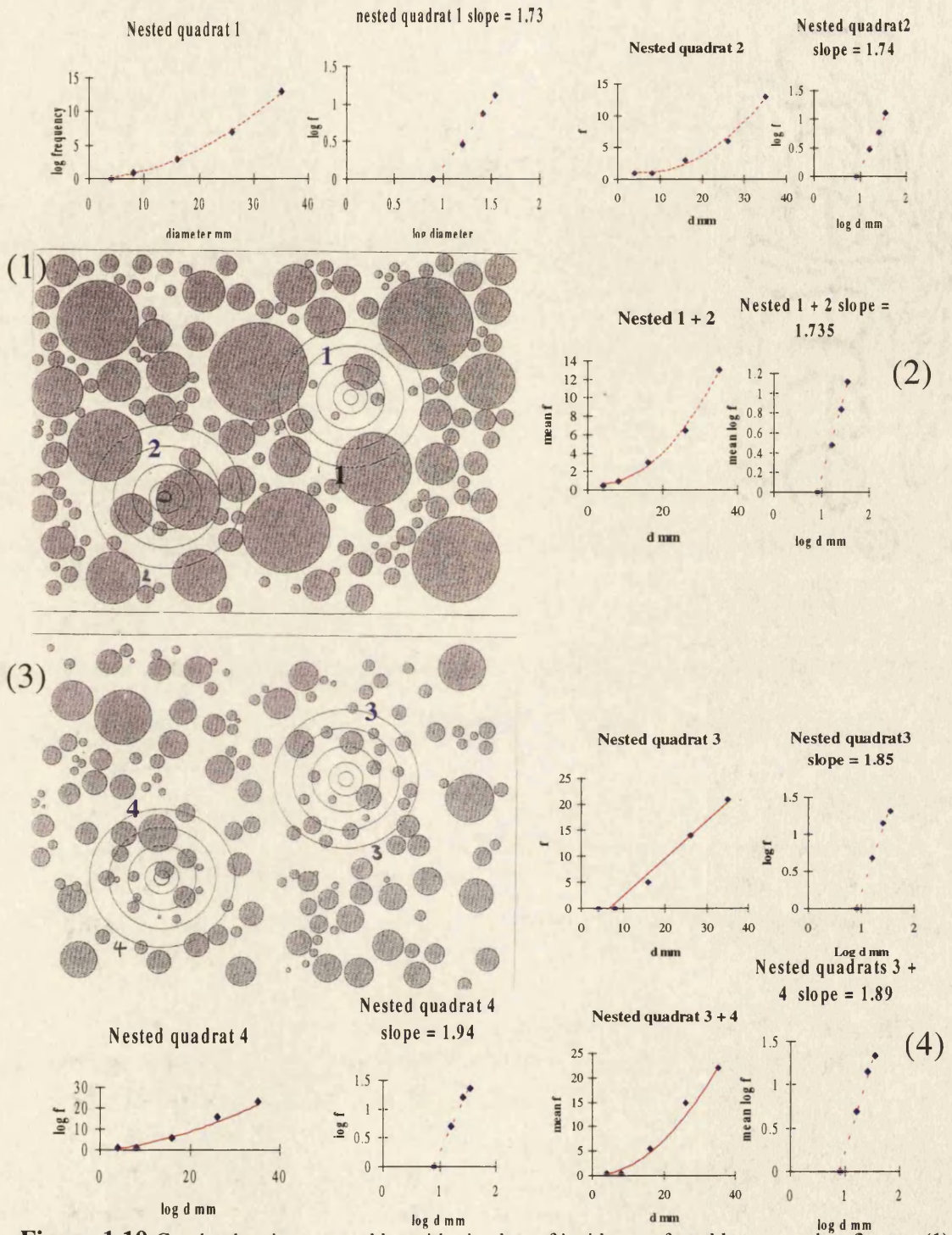


Figure 1.10 Graphs showing raw and logarithmic plots of incidence of marbles counted on 2 trays (1) and (3) vs. diameter from centre of 4 nested quadrats. The raw plots show an exponential increase in marbles counted with radius of search. The logarithmic plots to the right of each raw plot show straight lines with gradients which indicate the fractal dimension of the pattern in the two trays. Combined plots from the two nested quadrats in each tray (2) and (4) thus collate an average fractal dimension. Tray (1) shows a lower fractal dimension (2) than the fractal dimension (4) for the second tray (3).

1.8 Effects of heterogeneity in ecosystems, multiple-scales and perspectives

Living systems are composed of nested hierarchies of elaborately enfolded and intermittently touching boundaries which allow environments, as spatial domains of varying scale within the system, to be partially enclosed and also inter-connected. Across such boundaries, and along any paths of connected spatial domain, communication feedback loops operate, creating changes in pattern, which can be viewed at different scales. Trees, hyphae, xylem vessels, mitochondrial christae, chloroplast thylakoids and leaf veins exhibit good examples of branching patterns produced. These patterns are able to fill space in a way that maximises the amount of boundary (Williams 1997) in a field of view. Such type of space filling is most efficient when it has a high fractal dimension (West & Deering 1995, Ramsdale & Rayner 1997). The patterns created by viewing, for example, fungal genets underlying two forest leaf litters, are shown in figure 1.10. A method designed to overcome limitations of a fixed scale of view is nested quadratting (Rayner & Boddy 1998). Nested quadratting is an easy way to obtain the fractal dimension or a value for the efficiency of space filling that any distribution of boundaries has achieved. The two woodland sites are shown with two nested quadrats in each. The upper pattern (1) has many more sizes of genet, some of which take up large areas of the soil, whereas the lower pattern (2) is just as variable in genet size, but there is absence of very large size classes of genet. If the lay-out of each pattern is viewed through the nested quadrat range of concentric views numbered (1)-(4), and plots made of the diameter of view horizontally against frequency of genets vertically, exponential increases in the number of genets found can be seen. If the radius of view doubles there is a squaring of the number of genets observed. This relationship is similar to island biogeography theory. If plots are made of the same data but on logarithmically scaled axes we can obtain straight lines from which slopes can be derived. These slopes yield comparative values that are equivalent to the fractal dimension of the distribution patterns measured. Incorporating such nested quadratting techniques into a standard macro-fungal mapping protocol could enable a comparison of the types of diversity in terms of efficiency of filling space (fractal dimension). The fractal dimension of a system is functionally linked to the amount of boundary a system produces (or enfolds, divides or branches) into a given space. Difficulties in appreciating fractal organisation in ecosystems lead to conceptual traps and fixed measurement criteria. These conceptual traps (West & Deering 1995, Williams 1997) arise from approaches that effectively treat the system as a set of discrete compartments that can be sampled, analysed and interpreted without reference to one another, without relationship or interaction.

The development of a multiple-scale, rapid macro-fungal distribution mapping technique may lend itself to studies where laboratory and field work can be combined at several scales, for different research and environmental impact assessment requirements (Guevara & Dirzo 1998, Cannon & Walker 1996, Cannon 1998). Such an integrated approach which incorporates both nested quadratting and the rapid macro-fungal mapping protocol, will form the basis of chapter 3, and frame the discussion in chapter 8 on the effects of insect larvae on fungi and *vice versa*. Each scale will shed its own light on processes involved in distribution and pattern formation between wood-decay fungi and the mycophagous insect larvae that inhabit them. **Firstly, chapter 2 will consider the development and applicability of a standard rapid macro-fungal diversity survey technique for use in analyses of multiple-scaled fungal distributions using nested quadratting and abiotic factor fluctuation monitoring techniques.**

CHAPTER 2: MAPPING MULTIPLE-SCALED ECOSYSTEMS

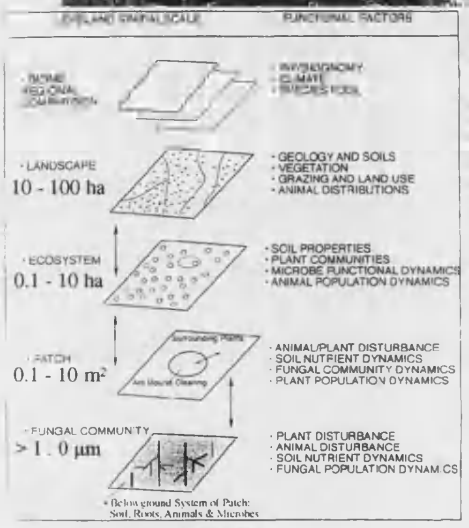
2.1 Synopsis

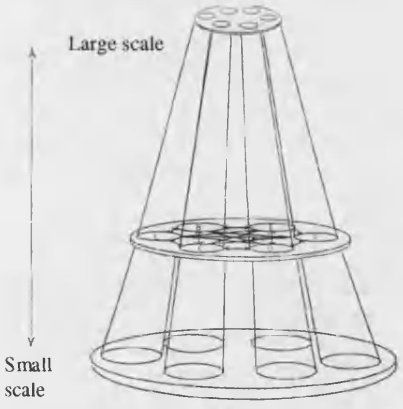
Following the first chapter's introduction to key themes, concepts and the organisms to be studied in this thesis, chapter two follows by briefly considering the fungal-biodiversity research fieldwork methods that I have devised. I developed these methods in order to tackle the issue of scale-plurality in forest ecosystems, particularly to compare vastly different forest sites in meaningful ways. The method consists of a standard, rapid mapping technique for macro-fungal diversity and abundance. Margalef biodiversity indices are incorporated to enable a comparison of fungal biodiversity between sites. Maps enable the structure of the fungal communities to be viewed over time and for changes to be tracked. This chapter considers in what way the standard mapping protocol which I developed could be adapted for the purpose of field work regarding insect-fungal relations in dead wood, by taking account of heterogeneity, scale and spatiotemporal dynamics.

2.2 Introduction

In studying any complex system, it should be decided whether to study multiple factors in parallel or to concentrate on just one or two aspects in isolation. The former "big picture" enables a synthesis of knowledge and therefore potentially enhanced understanding over the latter approach, but there is a risk of small sample sizes and therefore a lack of conclusive data. The latter enables large sample sizes and conclusive data, but only a limited view of the system investigated. Moreover, it is easy, with little knowledge at the onset, to launch into investigations choosing inappropriate factors, or inappropriate scales of observation. With only a limited conceptual view of a system, discussing the relevance of any statistical findings on ecological patterns and processes is very hard given that ecosystems organise themselves simultaneously at many scales. A multiple-scaled investigation leads to a more general and stronger understanding in which later specific statistical findings from isolated scales can be discussed more meaningfully.

The next decision regards methods to be used. The understanding of an ecosystem relates to the scale of observation and data collection protocols in the chosen habitat. This poses a potential problem of observation-scale-bias. For example, when visiting a novel forest habitat for the first time (figure 2.1) do we climb through its boundaries at local scales or do we view its entire structure from above (Cannon & Walker 1996)? Habitats consist of inter-taxonomic relations through the touching body surfaces of living systems. Living systems consist of nested hierarchies of sometimes elaborately enfolded boundaries that define at least partially interconnected spatial surfaces of widely varying form, texture and scale (Laurence 2000, Levin 1999, Wilson 1992 a). Difficulties in describing, understanding and conserving such systems arise from approaches which effectively treat the system as a set of discrete compartments which can be sampled, analysed and interpreted without relation to one another, with no interactivity or feedback between them (Cannon 1998, Kirby 1988). The problem with the variety in scale and heterogeneity that occurs in complex systems, is illustrated in figure 2.1. This figure shows scales with which one might consider the same ecosystem and the types of questions that might be answered at these scales.





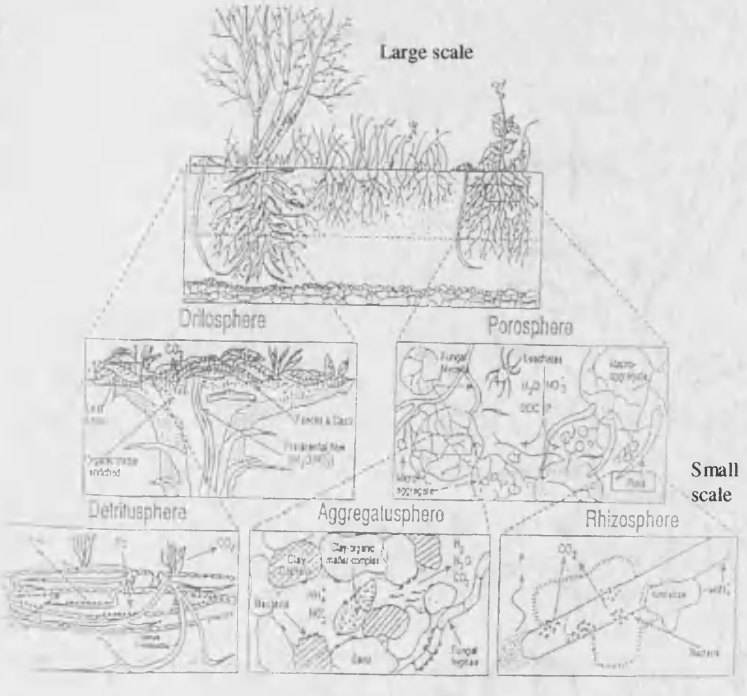


Figure 2.1 The issue of scale in studying any living system is highlighted by, at top the choices of study scope available when encountering a habitat for the first time, middle left; actual ranges at which ecosystems and their components simultaneously operate, below left, conceptual ways of viewing ecosystems as nested hierarchies of scales linked by the ecological processes which connect them, at right; actual fractal organisation of soil dwelling organisms from roots of large plants to the smallest microbes living between surfaces of charged colloids. (from Allan & Hoekstra 1992)

A further problem compounds the importance of the issue of scale. Some organisms, like fungi, exist simultaneously at scales from micrometers to kilometres, so answering questions meaningfully involves considering a multitude of scales at the same time. To overcome these difficulties in the study of fungi it is necessary to develop a more integrative investigative approach that allows the relationships between different spatial scales to be described. Two attempts to deal with this problem are discussed in this chapter: first, categorising macro-fungal abundance and diversity in Canada during a short three-month study period on the environmental impact of intensive forestry practices on the enclosed Fundy National Park, and secondly in assimilating macro-fungal biodiversity survey techniques in a three-month study of a Somerset woodland called "Inwoods" near Bath, UK. Lessons from both short-term studies were used to develop investigation methods for the 3 year study period on the relations between fungal-insect communities inside rotting wood (chapter 3). For this latter study, the technique of rapid macro-fungal mapping presented in this chapter was incorporated into a nested quadrat approach to take account of nested scales and to detect fractal dimensions (chapter 1). Fractal dimensions are derived from measurements of distribution patterns within heterogeneous spatiotemporal-dynamic ecosystems. This chapter therefore introduces and discusses the principal methods of fieldwork that were used to obtain the results presented in chapter 3.

2.3 Methods

2.3.1 Margalef biodiversity index

A method to rapidly map macro-fungal diversity and abundance was first devised in Fundy National Park in the Atlantic maritime forest of eastern Canada during a three-month environmental impact assessment (Taylor 1993). This standard method was later improved in a three-month local field-site study near Bath, UK (Taylor 1994). Once data had been obtained, abundance and diversity lists were obtained and compared between sites (Taylor 1993, 1994). Margalef biodiversity indices were calculated for the UK study but not for the environmental impact assessment in Canada. Thus calculations of biodiversity indices for the Canadian data, and comparison with UK "Inwoods" data, are presented here for the first time. The chosen Margalef biodiversity index has the advantage over other indexes in that it places equal weighting on both species richness and their abundance using the formula below (Margurran 1988).

Formula for Margalef biodiversity index (Dmg):

Dmg = (Total no. species - 1) / Ln total no. individuals

2.3.2 Fundy National Park, Canada

Four different sites were chosen representing some of the diversity within the greater Fundy ecosystem for a three-month study (Taylor 1993). Two sites in Fundy National Park were used as "Reference sites" since they were closer to the original types of mature, boreal-temperate-mixed, second-growth habitat which covered the region before intensive forestry practices of clear-cutting and plantation management spray-programmes (insecticides and herbicides) were introduced. Reference one (R1) was composed of a sugar maple (*Acer saccharum*), paper birch (*Betula papyrifera*) and gray birch (*Betula populifolia*) dominated stand, and Reference two (R2) was composed of a ceder (*Thuja occidentalis*), Eastern white pine (*Pinus strobus*), Scots pine (*Pinus sylvestris*), red spruce (*Picea rubens*) and white spruce (*Picea glauca*) dominated

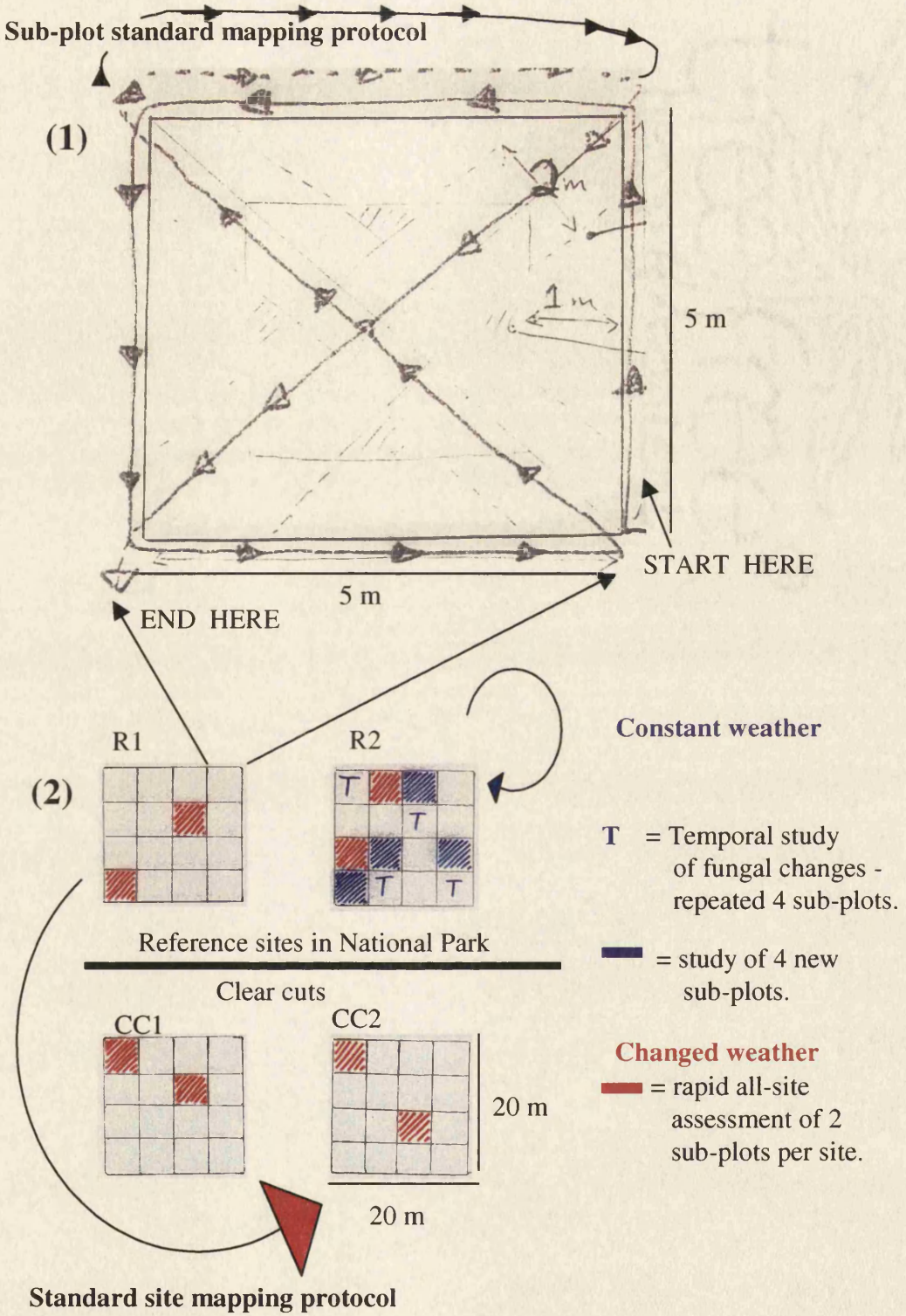


Figure 2.2 A standard macro-fungal mapping technique, using fruiting bodies as indicators of fungal genets, developed to compare fungal diversity between sites. (1) shows a single plot of 25m^2 traversed around margins and diagonals to map macro-fungal abundance and diversity. (2) shows each plot within 4 study sites of 400m^2 and how, depending on the weather, two types of sampling strategy were employed for intra-site temporal data (blue) and inter-site comparative data (red).

stand. Two sites were chosen in the clear-cut and intensively managed areas just outside the park: clear-cut 1 (CC1) was composed of a recent clear-cut with woody debris amongst planted Norway spruce saplings (*Picea abies*), and clear-cut 2 (CC2) which was composed of a 20 year old clear-cut, now an intensively pesticide-managed Norway spruce plantation (Taylor 1993).

The standard macro-fungal mapping technique that I developed is outlined in figure 2.2. Four grids 20 by 20 metres in each habitat were marked out and sub-divided each into 16 plots of 5 by 5 m (Taylor 1993). Two sampling strategies were used in combination to map any visible macro-fungi. Firstly, shown in blue, if weather patterns were roughly constant, I visited one habitat per day and chose 4 plots which had not been mapped before (shown as hatched blue squares) and 4 plots which had been mapped for monitoring and tracking temporal changes (shown as blue letter T's). The second strategy is shown in red. A short while after any noticeably large changes in prevailing weather conditions, a day was spent visiting all 4 habitats choosing two plots randomly at each to survey and record rapid fungal fruiting body flushes before they disappeared.

The standard way to map each sub-plot regardless of the habitat scale or structure was to start in the bottom right of each plot (top of figure 2.2) by carefully walking around the plot's perimeter facing inwards and directing my search one meter inwards using a one meter rule. I repeated this process walking diagonals sweeping the 1-m rule in two-meter wide arcs. When a tree was reached it was scanned as far up as I could see. In the centre of each plot weather conditions were noted and the overhead canopy cover measured. In this way little disturbance was created whilst maximising search efficiency in terms of area searched - see Taylor 1993.

To identify species mapped, I used a small mirror to look at the underside of gills without having to pick the fruiting body from mycelia. This minimised the survey's ecological impact and meant that subsequent maps of the sub-plot could be made to show temporal changes. Field identification books were used to identify genus or species if possible (Taylor 1993).

To optimise speed and practicality I decided to make a standard error of estimating groups of agarics to be of different genets if, when arising from soil, they were greater than 50 cm apart. Similarly if fruiting bodies were on separate logs of more than 50 cm apart I assumed likewise. Though I appreciate this was a large over-simplification, it facilitated rapid data collection in standardised fashion, enabling different habitats to be compared with ease where no such fungal biodiversity protocol existed before.

In order to check whether the plots mapped showed a good representation of macro-fungi, I complemented this standard mapping technique with wider ranging and more conventional fungus forays around habitats of each of the four sites. Notes were made regarding tree species, size of trees and canopy cover, other fungal interactions between taxa, for example, whether fruiting bodies were infested with insects (see Taylor 1993) or showed signs of having been eaten by molluscs or small mycophagous mammals. Care was taken by timing each foray to spend the same amount of time searching each habitat. Flagged trails to get to the study sites provided an opportunity to walk the same trail each day and look for temporal changes. An overhead densometer reflector was used to collect data of ground-level vegetation cover. This was done at each site at four central sub-plot outer corner markers to give indications of amount of cover and shelter offered to fungal fruiting bodies and mycelium in the leaf litter.

2.3.3 "Inwoods" woodland, Bath

Three sites were chosen for a three-month biodiversity-method-integration project in a Somerset woodland called "Inwoods", close to the University of Bath (Taylor 1994). Diversity and abundance of macro-fungal species were surveyed in three 20 by 20 m (400 m^2) study plots within three woodland habitats of the Avon valley ecosystem using the rapid macro-fungal standard mapping technique described in Taylor 1994. The three habitats chosen were a storm-damaged but regenerating habitat of beech (*Fagus sylvatica*) (S), a managed coppiced oak (*Quercus cerris*) and hazel (*Corylus avellana*) habitat (J), and an undisturbed hazel (*Corylus avellana*), beech and pine habitat (W). At the same time, five wood-decay fungal species, *Stereum hirsutum*, *Chondrsostereum purpureum*, *Phlebia radiata*, *Hypholoma fasiculare* and *Heterobasidion annosum* were used as homokaryon baits for two experiments of species-specific spore trapping.

Site S ("stumps"), was a storm-damaged, cleared, rapidly-regenerating deciduous habitat with many large deciduous stumps and pieces of large and coarse woody debris with high bramble cover. This site was part of a coppiced clearing approximately 50 by 50 m square (Taylor 1994). Site J, within the densest part of the woodland, was as far away from road and forest edge as possible. Site J was the most ancient piece of undisturbed woodland habitat, dominated by mature hazel (*Corylus avellana*) and large oak (*Quercus cerris*) trees, which formed the main canopy cover. Site W was a mixed pine and deciduous stand on a 20 degree slope of intermediate maturity between sites S and J in terms of developmental-succession towards a more climactic woodland ecosystem.

Using the same standard mapping protocol outlined in figure 2.2, relative macro-fungal alpha diversity (within-habitat species diversity and abundance - as in Magurran 1988) was quantified by using the standard fruiting body mapping protocol. Macro-fungal fruiting bodies were mapped and identified without removal of fruiting bodies from their original positions within plots. If fruiting bodies were needed for further identification, they were removed from outside study plots.

Fruiting body genets were mapped rather than individual rammets (by which I mean that multiple fruiting bodies connected to the same underlying mycelium were not mapped separately). Ground dwelling clumps of fruiting bodies were classed as separate mycelial genets (genetic individuals) if found on clearly separate resource blocks of wood and other substrates or if separated by more than 50 cm if found in soil. The mapping was also 3-dimensional by mapping positions of fungal fruiting bodies in the leaf litter and resource blocks of decaying wood, nuts and galls as well as fruiting bodies on tree bark surfaces up to approximately four meters in height (beyond which identification and mapping of fruiting bodies was difficult without the aid of tree-climbing equipment). Identification was carried out using field guides (Phillips 1981, Buczacki & Wilkinson 1989, Ellis 1976, Findlay 1977 & Lincoff 1981). A small mirror was used to look at fruiting body gill surfaces from the underside. Visual identification of fungal species was made on the basis of as many of the following as possible: fungal fruiting body morphology, habitat type, fruiting body size, texture, smell, spore colour and oxidative colour changes (Taylor 1994).

A way of minimising ecological disturbance to fragile soil-dwelling mycelia whilst surveying each fungal-plot had to be developed so that temporal mapping could occur at each fungal plot on successive occasions. Disturbance minimisation involved setting up strict walkways around and within each fungal plot using the borders of plots and plot diagonals. Whilst walking on plot borders the ground and trees were scanned visually using a meter rule as an aid to gently lift aside ground vegetation and leaves to search underneath. Whilst walking plot diagonals, however, the searching was more widespread to cover the remaining areas of

the plot but without feet leaving walkways. Furthermore, at all other times walking within study sites was restricted to fungal-plot diagonals and borders in order to minimise moving positions of fungal colonised woody debris or trampling of fungal fruiting bodies and ground vegetation.

All trees within each fungal plot were mapped and measured for circumference (cm) at breast height. Anything less than 5-cm circumference was considered a sapling. Trees were also identified with a field guide (Mitchell 1974 & Pokorny 1994). The canopy height of each plot was measured using a protractor and a plum-line suspended from its centre of radius. Five measurements of angles to tops of canopy trees were made at each site: four from corners of study sites to trees at opposite corners, and one from the bottom right corner to the canopy heights. The tangent of the angles from the slope of the ground to the tops of the trees overhead was multiplied by the distances from bases of the trees to calculate height. This was added to height of the eye (1.7 meters in my case) to give height of canopy above ground. Slope of ground was estimated by ensuring that the angle measured was between a line parallel with the slope of ground and tree canopy.

2.4. Results

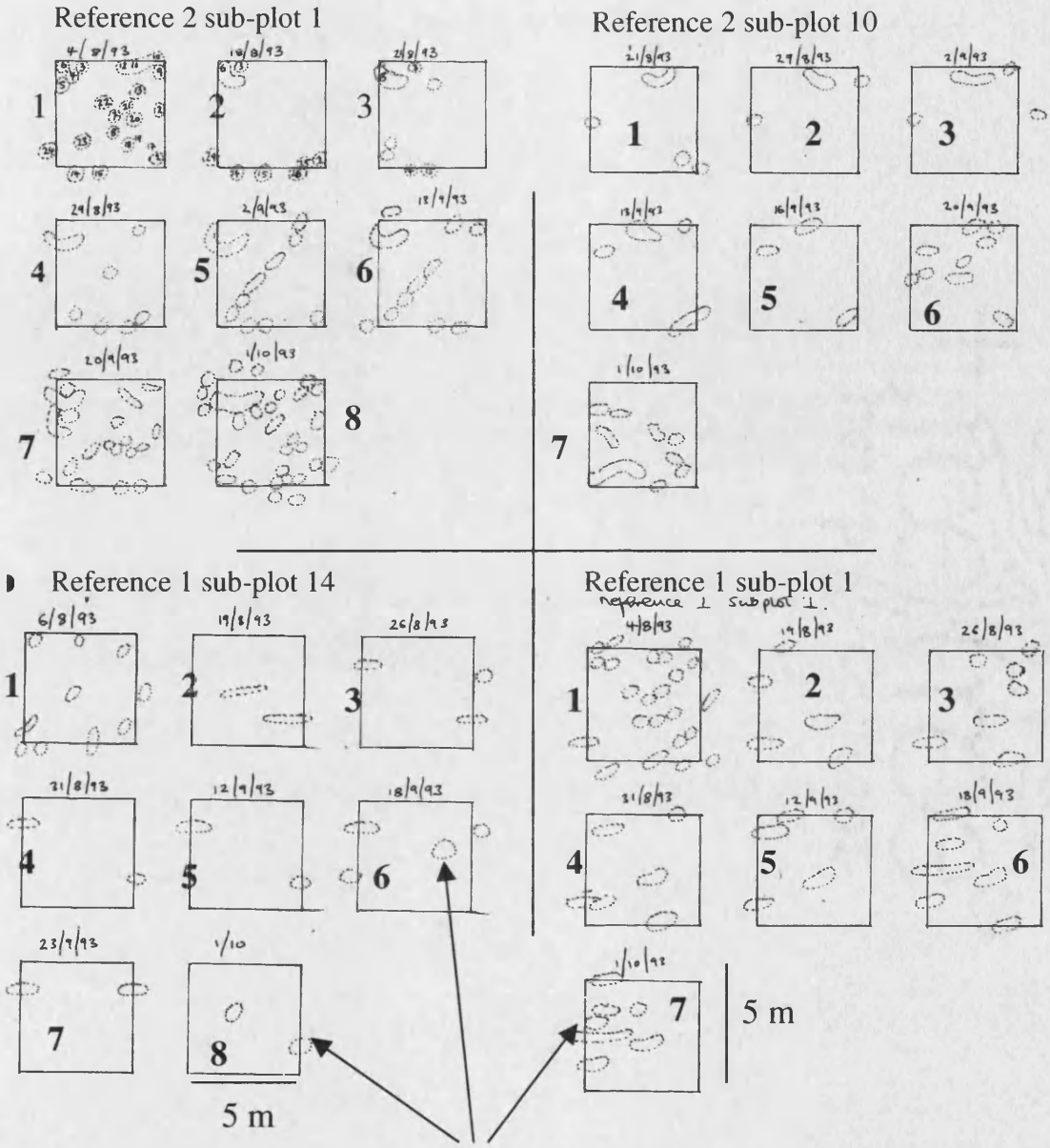
2.4.1 New calculations from Fundy National Park

Table 2.1 shows a new analysis of abundance and diversity data (Taylor 1993) collected from the 4 Fundy habitats. Reference site 2 shows the highest diversity with 257 mapped fungal genets comprising 66 macro-fungal species whereas clear cut 2 shows the lowest diversity with 27 mapped fungal genets comprising 12 macro-fungal species. Reference sites showed average mean of 235 genets and 64 species per site whereas clear cuts showed significantly less - with means of 112 genets and 21.5 species mapped (Taylor 1993). Margalef biodiversity indices confirmed these trends.

TABLE 2.1 Margalef indexes comparing diversity and abundance data

Reference 1 Margalef index =	**11.4**
Reference 2 Margalef index =	**11.7**
Clear Cut 1 Margalef index =	**5.7**
Clear Cut 2 Margalef index =	**3.4**

Figure 2.3 shows a new temporal mapping display of fungal genet dynamics in 4 sub-plots from the two reference sites in the national park. Some species such as *Xylaria longipes* and *Xylaria polymorpha* were present for the entire duration of the study. Others such as *Boletus, Collybia* and *Mycena* species formed fruiting bodies for only a few days, and then disappeared - triggered by changes in weather, most probably due to the abiotic factors of humidity, rainfall and especially rapid decreases in temperature. Each mapped square represents the same sub-plot mapped temporally from mid summer to late autumn.



estimated size of mycelial genets and their temporal positions
(from measuring around fruiting body clusters).

Figure 2.3 Temporal macro-fungal fruiting body dynamics; Maps of 4 Fundy National Park sub-plots in sites Reference 1 and Reference 2 during the Canadian study. Each 25m^2 sub-plot is mapped up to 8 times using a standard mapping protocol.

It is a testament to high rates of inter and intra-species fruiting body turn-over, that such dynamics can be mapped even over relatively short times in small 5 by 5 m patches of forest. The assumptions behind using visible fruiting bodies to estimate positions and sizes of underlying mycelial genets are discussed in the text. One benefit of using such a simple standard technique is that quite substantial maps can be drawn quickly to compare sites without access to a laboratory.

2.4.2 New results of work at "Inwoods" near Bath

Regarding average trends from mapping fungal macro-diversity (Taylor 1994), plot W contained an average of 10.0 fungal genets and 6.4 fungal species per sub-plot, plot J contained 5.0 fungal genets comprising 3.4 fungal species per sub-plot, and plot S had only 4.8 fungal genets comprising 3.3 species per sub-plot. In total, 40 species were found. Plot W had the most diversity with 27 species, whereas J and S had similar diversities with 15 and 16 species respectively (Taylor 1994). **New Margalef diversity index calculations are shown below in table 2.2 .**

TABLE 2.2 Margalef indices - macro-fungi of "Inwoods"

"INWOODS" study sites 1994	"INWOODS" study site 1995-2000
Site W = 6.12	**cecid study site =10.14**
Site J = 3.97	
Site S = 3.96	

With regard to the diversity and abundance of vegetation (Taylor 1994), 6 tree species were found at site W. These were ash (*Fraxinus excelsior)*, Scots pine (*Pinus sylvestris*), beech (*Fagus sylvatica*), Hawthawn (*Crataegus monogyna*) and hazel (*Corylus avellana*). Site J was found to contain 3 tree species: Turkey oak (*Quercus cerris*), hawthawn (*Crataegus monogyna*) and hazel (*Corylus avellana*). Site S possessed only 2 tree species: beech (*Fagus sylvatica*) and hazel (*Corylus avellana*). **New Margalef biodiversity index calculations for ground vegetation species are shown in table 2.3.**

TABLE 2.3 Margalef indices - "Inwood" ground vegetation

Site J = 2.1
Site W = 1.1
Site S = 1.1

2.5 Discussion

The rapid standard method for fungal biodiversity assessment as used in Fundy and near Bath can be used at different times of the year or throughout the year in terrestrial forest ecosystems. The results from Canada and the UK indicate that the standard mapping protocol may be used to obtain meaningful comparative data. Margalef biodiversity indices of 11.4 and 11.7 in Fundy National Park were much higher than those near Bath of 6.1 and 4.0, which reflects a history of heavier forest disturbance in the UK and the fact that Fundy National Park sits on the boundary of two forest systems, temperate and boreal forest - increasing the likelihood of higher diversity in the Fundy ecosystem.

2.5.1 Assumptions made

During both the Fundy and UK application of this rapid standard macro-fungal mapping protocol it was assumed that by keeping to walkways I would not overly disturb the underlying fungal mycelia and fruiting bodies in each sub-plot. It was assumed that fungal genets sizes could be coarsely estimated by a standard method of drawing a 50 cm radius around each fruiting body or group of fruiting bodies. Though closely

fitting the actual genet sizes of a proportion of species, for which it was used, this would not have been an accurate reflection for the smallest mycelial domains (generally occupied by Ascomycetes), or the largest mycelial domains (generally occupied by Basidiomycetes). Therefore I propose the following recommendation to reduce error to those wishing to use this method by estimating genet size on the following basis: **for all Ascomycotina, Basidiomycetes of *Mycena sp.* and *Merasmius sp.*, use a <15-cm separation between fruiting bodies to map as the same genet. For other Basidiomycetes use a <1-meter separation between fruiting bodies to map as same genet, and for basidiomycete rhizomorph-formers use a <5-meter separation between fruiting bodies to map as the same genet.**

It was assumed that looking at fruiting body diversity could provide a good indication of relative fungal diversity between each sub-plot and site. It was also assumed that fungal fruiting bodies were in close proximity to the microhabitat that the mycelium was utilising. I assumed that breast height measurements of nearest trees would give relative indications of size, height and possible age of trees. However the age would not be precise as many seedlings are repressed in growth because of effect of shading from a closed canopy on the quantity of phytohormones.

Canopy fungi could not be investigated more than 4 m above ground level. Tree climbing equipment would extend such three-dimensional surveys vertically to include important canopy layers where at least half of all fungal decomposition takes place prior to the fall of woody debris (Boddy & Rayner 1984, Moffett 1998). **Surveys could be conducted all year round at permanent study plots to obtain long-term data** (Minter 1996, Franco-Molano 1998, Mitchell 1998).

2.5.2 Applicability of rapid macro-fungal survey technique

Margalef biodiversity indices, which weight equally the diversity and abundance of species within study sites (Magurran 1988), seem to offer good comparisons when used in conjunction with the rapid standard mapping protocol as developed in Fundy and near Bath. However, considering the assumptions on genet size, how meaningful were these results really? The method has its strengths in its ease and speed of use, its lack of expensive equipment or laboratory access requirements, an advantage when considering work in more remote ecosystems, and its ability to compare and contrast sites for their fungal β-diversity (Magurran 1988). The weaknesses of the protocol lie in its species identification methods and genet mapping precision - which could be improved (as recommended above). Spore trapping was successfully integrated into the standard mapping technique (Ingold 1953 + 1971, Rayner & Body 1998) though it seems from the results that spores may be viably conveyed through long distances by air currents between trees since there was little relation between trapped spores and the presence of that species in the study plots. This could differ in less fragmented forest landscapes where the effect of forest edges would be considerably weakened and spore rain would be more representative of local fruiting bodies. Alternatively, the distances between study sites may not have been great enough near Bath to detect significant differences in constituents of spore rain, this despite the between-site difference in Margalef indices for fruiting bodies.

For the purposes of both Canadian and UK studies, a species was defined as a population of an organism that will not normally mate to form fertile hybrids under natural biotic and environmental conditions (Wilson

1992 a, Mallet 1995, Claridge, Dawah & Wilson 1997) i.e. within its natural ecological niche. It was expected that, by looking for macro-fungal diversity of fruiting bodies using a simple and standard mapping protocol, an indication of the underlying diversity of fungal species would be comparable between sites. In this respect, it was possible to successfully compare sites in Canada with those in the UK. However, there are points that must be acknowledged before coming to any conclusions about the overall applicability of this rapid mapping protocol. Many fungal species have been shown to contain numerous sub-species and strains. *Heterobasidion annosum* has sub-species specialised to inhabit three tree families and *Coriolus versicolor* and *Stereum hirsutum* also consist of several sub-species. Morphology, even at the scale of spores, fruiting body and culture characteristics, may not be able to distinguish sub-species of fungi since these are so variable. To investigate at this scale one requires all the techniques of molecular biology. So genetics of mating type and their alleles would have to be incorporated into those biodiversity investigations of fungi which demanded 100 % accuracy of identification to the scale of sub-species. However, the criticism of fungal taxonomy based on DNA provided by Seifert *et al.* surely strengthens the case for a more user-friendly identification approach (Seifert *et al.* 1995). Whatever the scales we chose with which to filter the information available in a habitat, molecular to morphological, we are left with inevitable compromises regarding sources of error. The challenge therefore is to define the scales at which one can meaningfully work, and accept the diversity of data produced as equivalent and comparable.

To be sure of the identification of fungal species there is often a need to check certain features microscopically such as spore sizes, shapes and colours. Since there was not enough time, nor the facilities to do this practically for each of the many fungal species recorded in these field studies, it was inevitable that a simple method such as the one used here would not be 100 % accurate in terms of species labelling. Moreover the rapid standard mapping technique lends itself to projects wishing to rapidly compare diversity and abundance quantitatively and to track the temporal changes that occur in terms of turn-over, growth and genet-movement, where perhaps little or no previous knowledge was held regarding the fungal kingdom. The types of identification errors made by the rapid mapping technique were likely to be standard. Miss-identification errors of a particular species will not have affected the diversity and abundance results in their quantitative analysis meaning that inter-site comparisons can be made meaningfully. **What the standard mapping technique therefore achieves is a new rapid protocol to obtain comparable data on fungal diversity between any two or more habitats at any time of year.**

Any rapid and user-friendly field protocol, which can be used with little training, is a compromise between time availability, ease of use, speed of identification technique, replicability of the protocol, cost of materials - especially with potentially large sample sizes involved, and large numbers of plots to be studied and compared. The standard mapping protocol is a novel technique, which removes the subjectivity from usual fungal forays used up to now. It enables species diversity and abundance to be compared in a short study time. In conjunction with nested quadratting techniques to take account of heterogeneity, this technique was considered the best with which to embark upon a combined laboratory and fieldwork based project to understand **spatiotemporal relations between cecids and other insect larvae and fungal communities within decaying hazel poles.** I would like to now draw your attention to the following particular case study: organisms that live below bark in the sub-cortical zone of dead hazel wood. **How then do surroundings relate to the distribution of fungivorous insects, in particular cecid fungus gnats, and the structure of communities of wood-inhabiting fungi of deciduous hazel coppiced woodland?**

CHAPTER 3: OBSERVATIONS OF INTERACTIONS BETWEEN CECID AND FUNGAL COMMUNITIES IN NATURALLY DECOMPOSING HAZEL WOOD

3.1 Synopsis

The kind of multi-scaled approach discussed at the end of the chapter 2 is applied to a case study involving a fungal-insect relationship. This, as far as mycology is concerned, seems the most efficient way of answering ecological questions meaningfully. It also introduces a range of perspectives from which to view the distribution, ecology, development and taxonomy of insects. Insect and fungal communities are structured at the level of the landscape, the forest fragment, woodland floor, individual coppiced hazel stands, woodpiles and at the level of individual logs and cavities under bark. This chapter is essentially an exploration of the field habitat in which both sets of organisms have relationships, both to each other and to their local abiotic and biotic surroundings. Consideration is given to: ambient conditions under bark, log piles, standing trees, basic observations, cavity measurements, mapping techniques, biotic and abiotic partners. Through this interplay of perspectives, a synthesis is approached which relates the distribution and behavioural patterns of fungivorus insects, in particular cecid larvae, to the complex boundary configurations generated by communities of fungal mycelia in decaying wood piles in the field. It is this synthesis that will become important when interpreting laboratory results in later chapters.

3.2 Introduction

The species that I have investigated live principally under three layers of fungal colonised rotting bark of dead hazel (*Corylus avellana*) logs. In figure 3.1 can be seen bark lifting associated with *Vuilleminia commedens*, a Basidiomycete, and visible are fruiting body pustules of the ascomycete fungus *Hypoxylon fuscum*, the fungus with whose mycelium cecid larvae seemed to be particularly associated. However, from the onset of the project I re-encountered the same challenge as in chapter 2 (Taylor 1993, 1994, 1998 b): of different scales at which patterns and their underlying processes occur regarding the ecology of woody decomposition (Harmon *et al.*1983). So instead of choosing one scale, which would have reduced both scope and the options for further study, I worked simultaneously at multiple scales in order to answer the following basic ecological question: **"What factors influence the development and distribution of populations of cecid larvae?"** There were seven chosen scales (figure 3.2) which were combined through the principle of nested quadrat sampling, which, as introduced in chapter 1.7, can also be used to estimate the degree of nestedness, heterogeneity and the fractal dimension of distribution pattern (Ramsdale & Rayner 1997). This chapter presents results of work at the first six of these scales, ranging from a landscape view of log piles and hazel infected with both the wood-decay ascomycete *Hypoxylon fuscum* and interacting members of the basidiomycete community, especially *Vuilleminia commedens* and *Hymenochaete corrugata*. The fungal community interacts at these scales with cecid larvae, individual logs and 3 layers of bark and sub-cortical zone. Populations of cecid larvae and *H. fuscum* mycelial genets, fruiting bodies and pseudosclerotial plate (PSP) zones occur within the bark complex of *Corylus avellana*. Figure 3.3 shows cecids in this natural context.

Observations regarding cecids were made whilst mapping the study site during the rapid macro-fungal survey (chapter 2). The phenomena of cecid jumping was first observed in *Miastor metraloas* by Wagner in 1863, as reported by Gabritchevsky 1928, and also confirmed by later workers Springer in 1915 and Kahle in 1908 (Gabritchevsky 1928, Springer 1915, Kahle 1908). In this chapter I confirm cecid jumping in a different species of paedogenic cecid, identified through the first scanning electron microscopy of this species as *Brittenia fraxinicola*. Data on insect larval jumping in general, including cecid jumping, have not been obtained before now, as far as I am aware. Between April and August the field cecid populations declined. In this period of water stress, individual *B. fraxinicola* larvae took on a rigid and immobile form that lacked responsiveness to stimuli and which possessed thickened, crust-like outer cuticles as if they had been "fried and battered" (Figure 3.10 - 3 and 4). These were subsequently found to be dormant *Brittenia fraxinicola* hemi-pupae. Medullary rays from logs with more than one species of fungal decay were often observed to protrude under bark in areas where pseudosclerotial plate was extensive and the surrounding sapwood had rotted away. This provided a series of long spaces longitudinally under outer bark layers in which cecids, other dipteran larvae or pupae and many small invertebrates were found. Another interesting observation, made with Alan Rayner, was that a fungal mycoparasite *Calcan sporium* grew in waves along a log surface. The effect of successive wave fronts of its mycelium changed the colour of the hymenial surfaces of *Hymenochaete corrugata* colonies over which it passed from light brown to a much darker colour. Slime mould tracks were often observed to produce a similar effect on the colour of various fungal hymenial surfaces over areas of bark. A log was kept outside the Department of Biology and Biochemistry and monitored at intervals with *V. commedens* and *H. fuscum* interactions. In November and December 1997, cecids were observed to be feeding immediately under the rolled bark running parallel to the interface between fungal interactions. Rolled bark is a characteristic of *V. commedens* decay on hazel. A part of this log covered in plastic hydrophobic insulating layers was seen to have more cecids visible at times of dryness. At these times cecids were also found foraging on the underside of the plastic. Cecids were found deep within the soft-rot in the sub-cortical zones in non-covered areas of log in dry conditions. One of the field cecid populations was on a standing hazel pole that later blew down. Cecids here were only found in bark areas in which both *V. commedens* and *H. fuscum* were forming interaction zones. These zones were characteristically melanised and sclerotised areas of wood-decay. Over these areas of interaction bark seemed looser with up to 1 mm gap separating the outermost layer from underlying sub-cortical zones. The underside of bark in these areas was also often characteristically blackened.

Figure 3.4 shows a generalised body plan of the most common field cecid that was identified as *Brittenia fraxinicola*. This species was discovered in 1941 (Wyatt 1967). This is the third scientific study to involve *B. fraxinicola* and the first to consider the natural ecology of this species. Previous studies have considered taxonomy and the paedogenic life-cycle (Wyatt 1967). Microclimate temperature varied greatly in the study site over time and in location. Temperature is thought to be relevant to larval diapause entry (Leather, Walters & Bale 1993, Heinrich 1999, Charlesworth & Shorrocks 1980), the conversion of cecid hemi-pupae to thickly insulated resting mothers (Springer 1915, Nikolei 1958) and the development of insect eggs (Richards 1965). Therefore a limited investigation into the heterogeneity of temperature differences between sub-cortical and ambient conditions was undertaken and is included in this chapter. In addition, this chapter also discusses some important observations. For example, cecid larval "jumping" behaviour had previously been observed by Wagner in 1863, by Kahle in 1908 and noted by Springer in 1915, but has never been

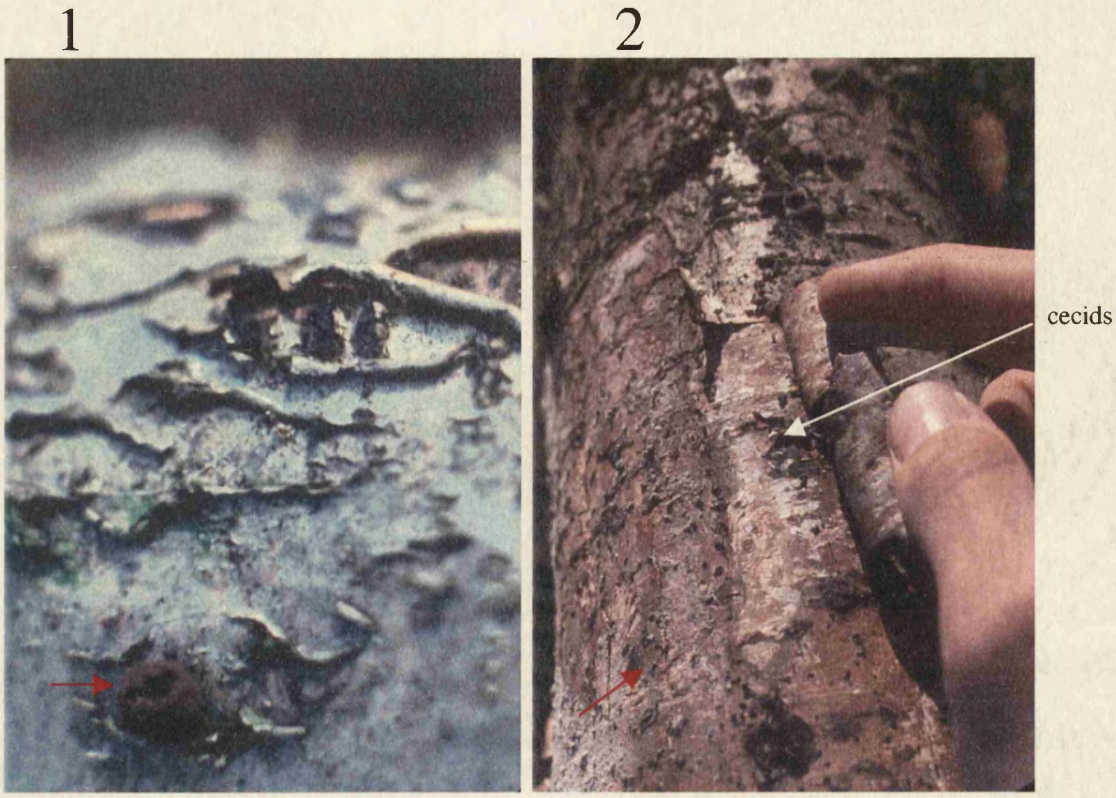


Figure 3.1 (1) shows the fruiting bodies of *Hypoxylon fuscum* (arrowed) indicating that this hazel log accommodates a decay column of the ascomycete fungus. Also bark cracks and lifting can be seen just in focus above a PSP zone interface between this fungus and others of the hazel decomposition community.

(2) shows the white, smooth and slippery when wet, fruiting body spore-producing surface of *Vuilleminia commedens* (arrowed); which indicates that this basidiomycete fungus resides underneath. It is characteristic for hazel bark to peel back up to the PSP zone between this fungus and other members of hazel's decay community; in this case *H. fuscum* whose fruiting bodies can again be seen above my finger.

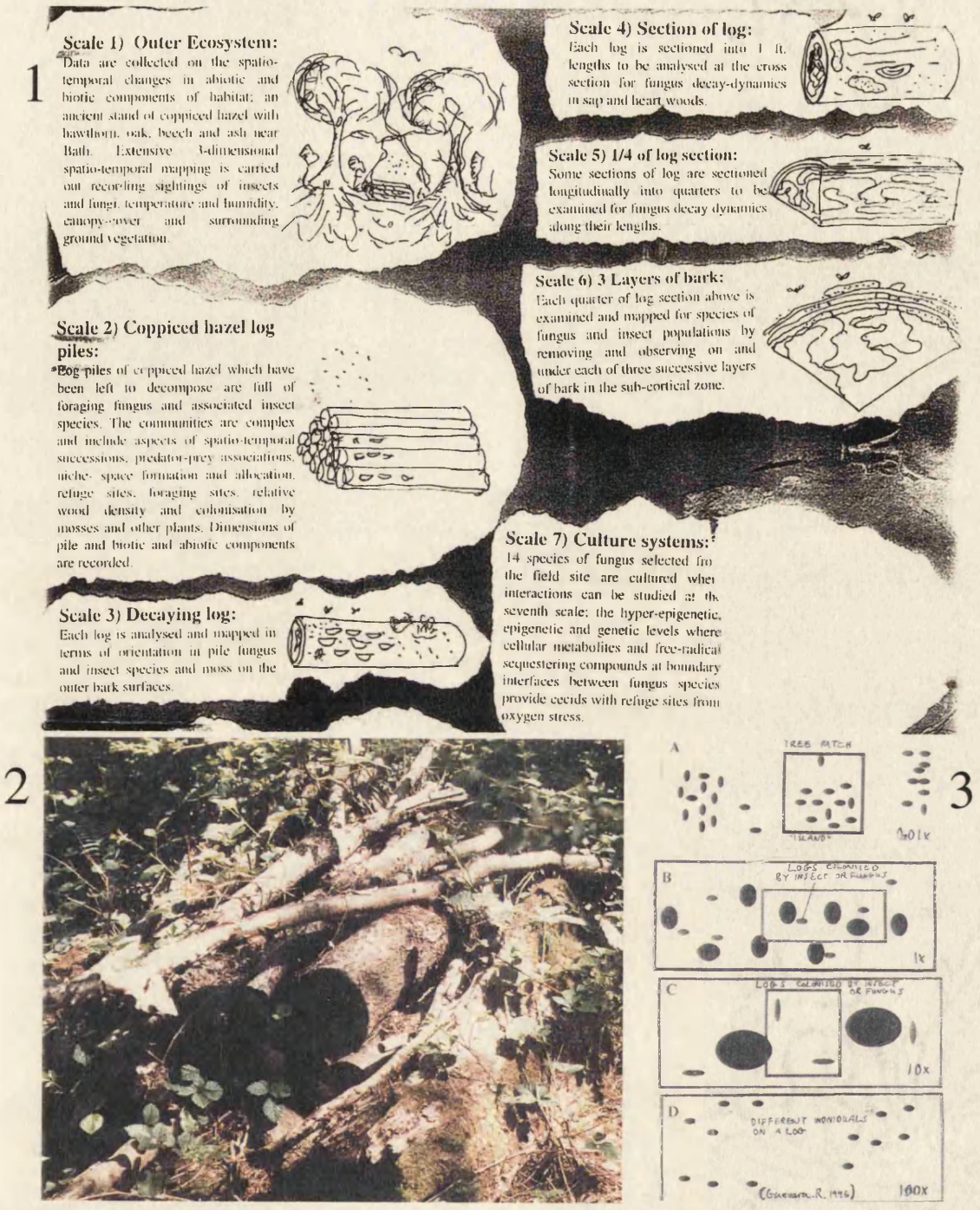


Figure 3.2 Combined strategies for ecological studies of nested scales represented by the schematic at top (1) where 7 independent scales are transgressed from observations and measurements in whole ecosystems (top left) to events in laboratory cultures (bottom right). Below this, at left (2) is a photograph of log pile 1 in plot 1 of the field site, And at right (3), a conceptual view of the fractal geometry of nested scales of ecological organisation such as those found in log piles within forests.

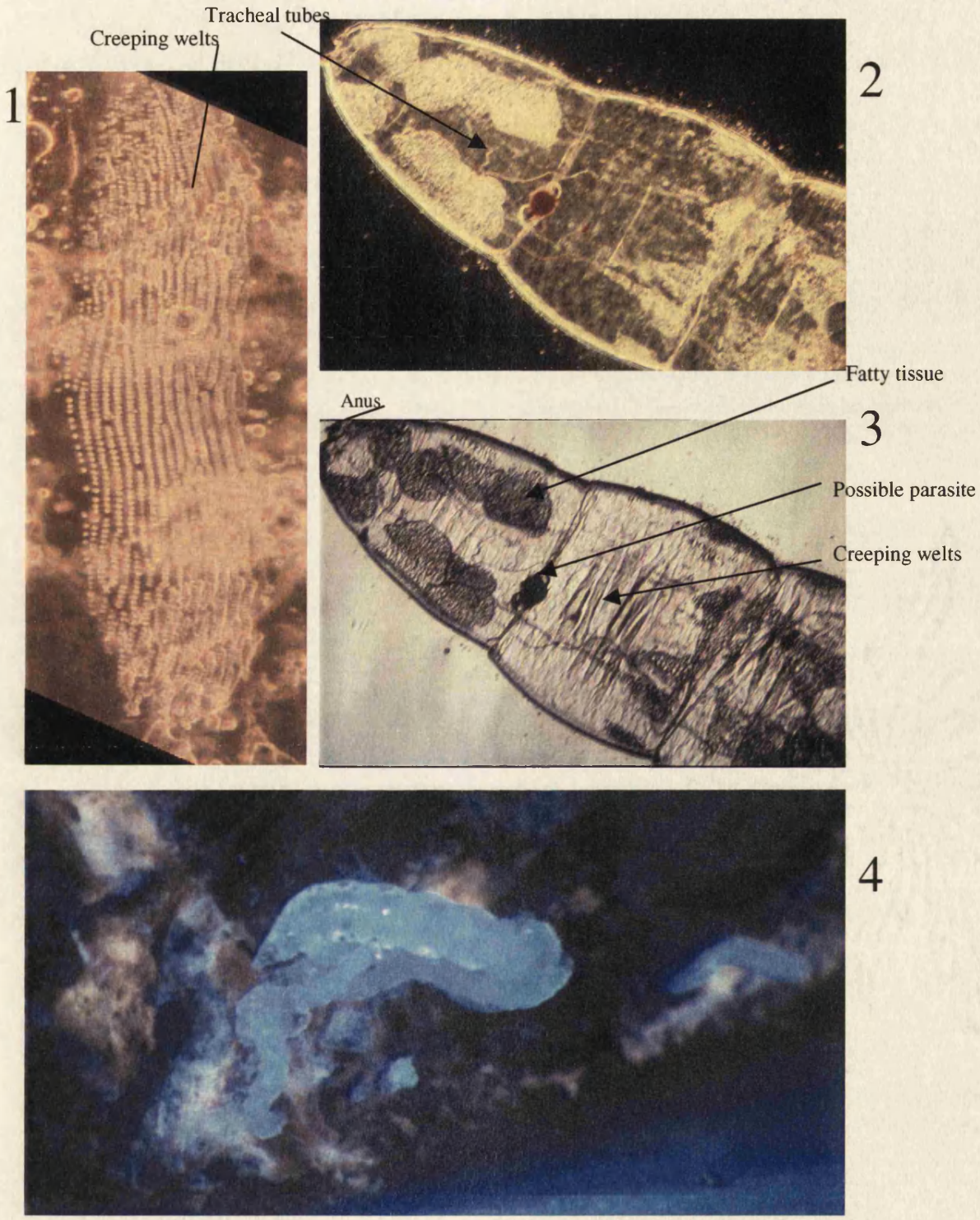


Figure 3. 3:Field cecids (1), (2), and (3) show polarised light microscopy under a yellow and white filter. (1)shows the ventral creeping welts just posterior to each of 10 larval segments. Top right (2)shows the posterior of the most abundant field larvae *Britennia fraxinicola* showing internal organs, fat bodies and creeping welts . The lower image (4) shows a range of sizes of this abundant cecid species as found on the under-side of lifted hazel bark. The species can also be found under bark of ash and birch (Wyatt 1964).

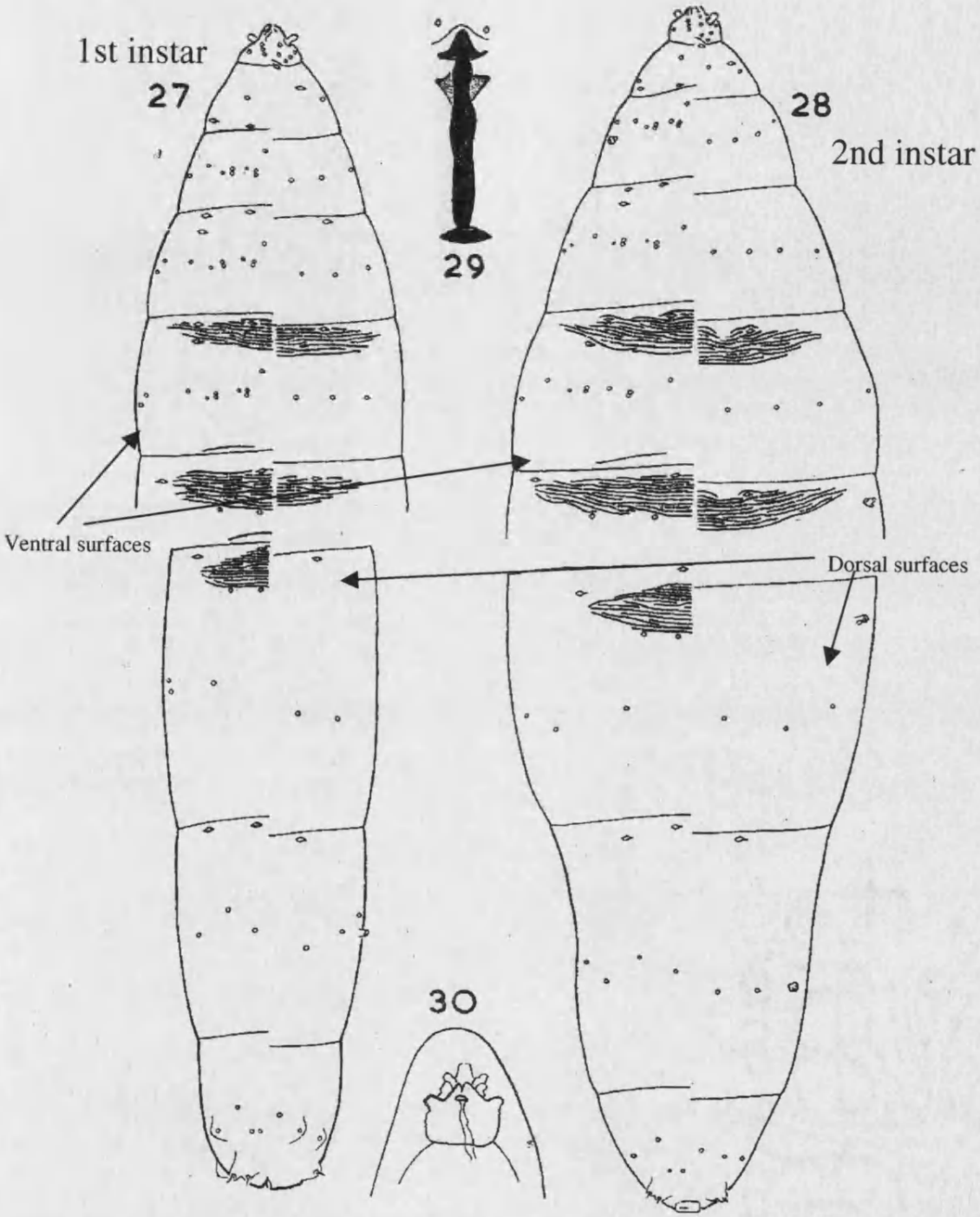


Figure 3.4 I.—*Brittenia fraxinicola* Edwards. Diagrams of papilla and spinule row arrangements of ventral (left) and dorsal (right) surfaces of head, thorax, first, seventh (typical), eighth and ninth abdominal segments: (27) first instar; (28) second instar; (29) sternal spatula; (30) anterior end of embryonic membranes. (taken from Wyatt 1967)

Key features of the most common field cecid *Brittenia fraxinicola.*

discussed in terms of dispersal strategy or in relation to spatiotemporal distribution pattern of cecids to their source-sink niches.

3.3 Methods

Initial fungus and insect forays were conducted in local forest fragments within the Avon bioregion with my two supervisors, Alan Rayner and Stuart Reynolds from November 1995 to January 1996. A field site was chosen in one of the largest intact forest fragments overlooking the Avon valley about six miles from the University (Ordinance survey pathfinders 1183 and 1184, co-ordinates 80, 65). The chosen field site (figure 3.5.) was particularly relevant to this study because it had a large number of piles of decaying coppiced wood, comprised predominantly from surrounding hazel but also some hawthorn, oak, ash and beech trees, reflecting the proportional tree composition of this habitat. Also, since I had studied parts of this woodland before for macro-fungal biodiversity, I had a good familiarity with this woodland (Taylor 1994). As a result of the age of the log piles, the decay communities were initially all at a similar and early-stage fungal community development at the start of this project and so could be monitored over a number of years. From these piles, log samples were sampled at intervals to piece together fungal and insect successions through the wood decomposition process. Also aspects of wood decomposition were correlated with size of pile and other abiotic factors. Thus the initial conditions in terms of positions of log piles and logs within them became of considerable importance relating to the direction of future events. After one of the piles was removed for firewood in the autumn of 1995, notices were posted on or near to log piles in the spring of 1996 asking the public, in the name of ecological research on decomposition processes, to refrain from removing dead wood. This successful open strategy enabled my weekly visits to the study site to continue to yield fruitful insights, perspectives and data.

3.3.1 Scale 1- Study site methods

3.3.1.1 Log piles and colonised hazel poles within landscape

The study site was divided up into three 20m by 20m plots. A fourth plot was 25m by 25m (fig 3.6). Each plot comprised sub-plots each of 5m by 5m in a grid system which was marked out with small green stakes. These four plots were gradually mapped using a nested quadrat approach for complex heterogeneous environments of multiple and interacting scales.

Sub-plots were mapped during the year to give temporal analysis of vegetation, macro-fungi, cecid locations and decay changes in log piles over changing seasons using field guides (Buczacki 1989, Corticuisse 1999, Phillips 1981, 1980, 1979, Ellis 1976, Findlay 1977, Lincoff 1981). This included mapping all trees with species and breast-height diameters on sub-plot data sheets. At the centre points of each sub-plot were recorded the following abiotic fluctuations: ambient humidity, ambient temperature, cloud cover, light intensity, visibility, soil pH, soil water content and soil temperature. This was followed by recording the biotic fluctuations of leaf-litter depth and tree-cover. There were two scales through which to record sub-plot data. Using a nested quadrat approach, sub-plots were first examined whole, and then in four $1m^2$ quadrats at midpoints of each 5 m sub-plot boundary. With each scale, the boundary of the square was searched up to 1 metre or 20 cm inwards respectively. Then diagonals were searched up to 1metre either side. Positions of fungus fruiting bodies and woody debris were mapped, as well as predominant types of ground vegetation. The species of moss were recorded if in the immediate vicinity to fungal fruiting bodies and mycelial decay

Figure 3.5 The field site chosen to study cecid-fungal interactions; 1: looking up at the site from the river Avon. 2; hazel logs in log pile three. 3 + 4 show the thin re-generating trees left by the coppicing work to provide a canopy. 5: log pile 10. 6; mixed hazel and ash stand at Northern edge of study site in which standing poles with substantial cecid populations were able to be found at most times of year.

columns on hazel logs of interest. Each macro-fungus species was mapped and identified as far as possible in the field and its position recorded. Samples of fruiting body were brought back to the lab for further identification if necessary. The mapping occurred from ground level up to 10 feet in height so that changes in habitat heterogeneity could be mapped three dimensionally. Log piles were mapped by numbering them and measuring width and length. Any insect adults, pupae or larvae seen and easily identified were noted as well as on what material they were found. Canopy cover was also recorded.

3.3.1.2 Local observation searches

Local observation searches were undertaken in woodland surrounding the plots, by viewing from leaf litter to 10 feet high. At sites where cecids were found, spatiotemporal notes were taken regarding position, condition, colour, morphology, size and ability of cecids to react to the stimuli of two touches with a fine artist's paint brush, to see if they were mature mothers or resting mothers and hemi-pupae (Mamaev & Krivosheina 1993, Wyatt 1967). Notes were made on species of associated fungus, colour, texture and wetness of fungi and sub-cortical wood.

3.3.1.3 Temperature readings

Temperature readings were taken using two thermocouple probes attached to a digital thermometer in sub-cortical layers where cecids were found and readings compared with ambient temperatures.

3.3.1.4 Larval colony situations

Other measurements of cecid populations included height from ground, diameter of woody debris, situation of woody debris (in log pile or otherwise), the orientation of wood and species.

3.3.1.5 Margalef diversity index

After accumulating diversity and abundance data for macro-fungi, the Margalef diversity index was calculated as in the formula given in chapter two.

3.3.1.6 Observations and notes

Observations relevant to this investigation were also made wherever insect larvae were discovered together with mycelium in rotting wood, plant galls, Bath University Garden's compost heap, in mushrooms, hollow mycelium-filled grass stems. Preliminary notes were taken in 1995 at the museum of forestry at the Forestry Research Institute, Dehra Dun, India.

3.3.2 Scale 2 - the log pile methods

Once a month one of 44 log piles was chosen at random, measured, drawn, and number of logs counted and recorded on a log pile data-sheet. Measurements of internal and external abiotic conditions were also recorded. These consisted of temperatures and humidities inside the middle of the pile above ground level, inside the middle of the pile at ground level and soil water content at ground level. Also sub-cortical temperatures of the pile were measured internally and externally and compared to ambient temperatures outside the pile. The pile was then dismantled and species of fungus and vegetation including bryophytes and pteridophytes mapped on each log. Logs with cecids and fungi were labelled carefully with pile number, log number and either destructively sampled *in situ* or brought back to the university for sawing up into sections.

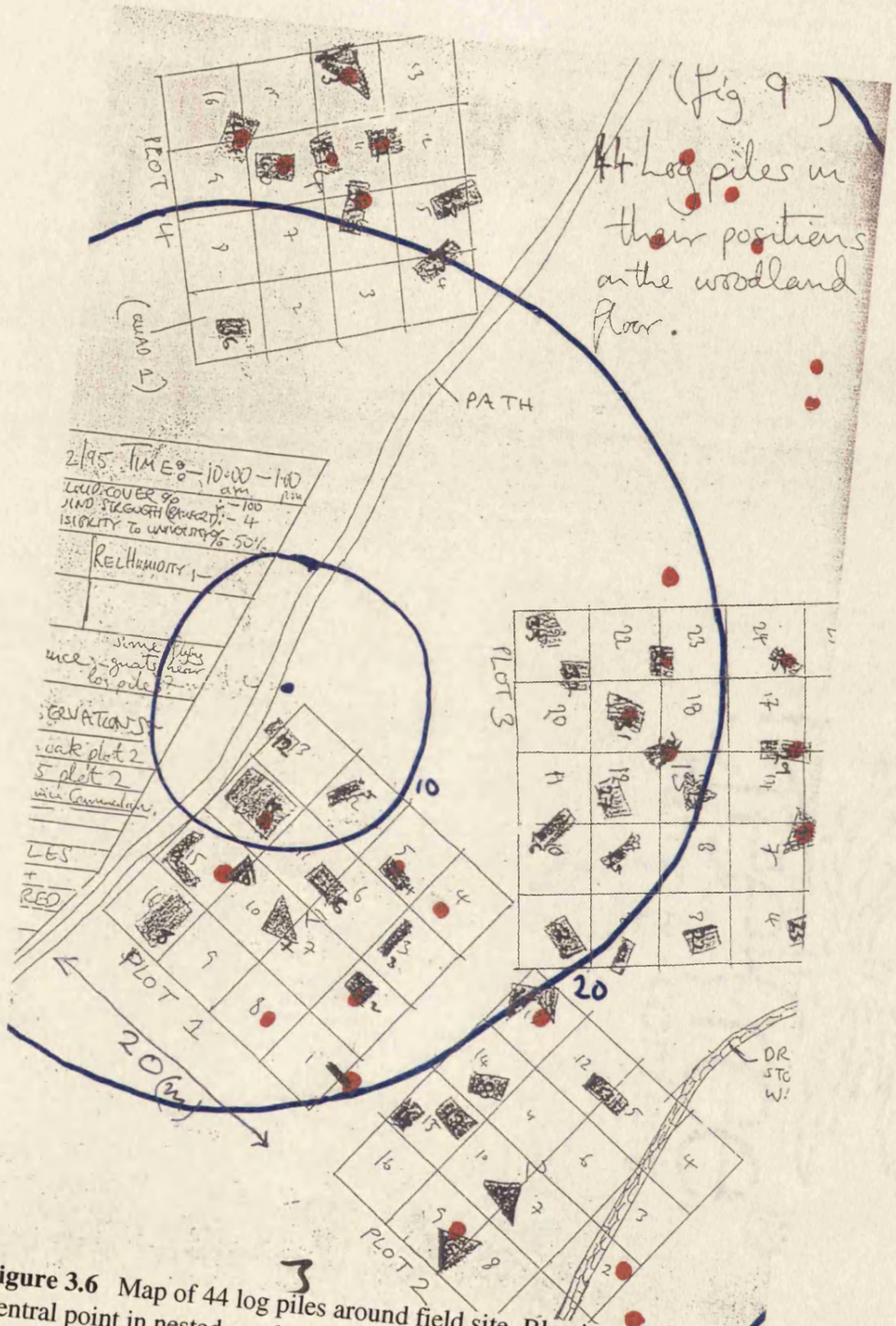


Figure 3.6 Map of 44 log piles around field site. Blue indicate distances from Central point in nested quadrat. Red dots indicate cecid populations found.

3.3.3 Scale 3 - Individual log methods

Thirty logs were measured in terms of length and diameter before being mapped for insects, fungi, moss, and diversity of species. Absence or presence of cecids under the bark was also investigated at four sample sites along the log. Bark was peeled in a 5 cm diameter patch from the underlying sapwood and cecid presence noted.

3.3.4 Scale 4 - Cross-sections of log methods

Logs were then sawn into lengths of approximately 50 cm. Each length was labelled carefully with both log pile and log from which it came. This was to ensure every observation could be traced back into the field situation from which it had come. Each log length was mapped for insects and fungal domains both at cut surfaces and by destructively removing sub-cortical zones with a small and well sharpened machete down to sapwood to dissect for presence or absence of cecids and other insect families.

3.3.5 Scale 5 - Longitudinal sections methods

Ten log lengths found to contain cecids were then sectioned with a band saw into four quarters longitudinally, tied together, labelled and placed in a black plastic bag. Bags were placed on some woodland soil under the trees behind the Department of Biology and Biochemistry, University of Bath. Each cross-section was then examined and mapped for fungal domains and species and cecid positions, placed back in the bag to continue decomposing, and looked at again after six months.

3.3.6 Scale 6 - Log cross-section methods

Examination primarily for cecids but also other insects and invertebrates such as mites, Collembola and nematodes took place by sequentially peeling back each layer of bark and looking at both upper and lower surfaces under a binocular microscope, as in a cross-section. The species of wood was noted before dissection and a record kept. Hazel (*Corylus avellana*), the predominant wood type in the study site, has three layers. The first two were inspected above and below, as they could be peeled or stripped easily off with a blade, whilst the third innermost layer was inspected at its top surface. Observations were recorded and any cecids found had positions noted accordingly in terms of vicinity to *Hypoxylon sp.* ascmomycete fruiting bodies or basidiomycete mycelial chords and fans in the sub-cortical zone.

3.3.7 Field emergence trap methods

In addition to destructive sampling of logs for larvae and fungi, trapping of emergent adult insects from individual logs took place between spring 1998 and summer 1999 (adapted from Owen 1989, 1992). A combination of pictures and keys in Chinery 1986, Sterry 1997, Hutson 1980, Freeman 1983, Chandler 1988, 1992, were used to provide identification of orders and insect genera.

3.3.7.1 Sewing the traps

Traps were made from the finest nylon gardening mesh with holes of less than 0.5 mm^2. They consisted of a rectangular piece of mesh, 0.75 m by 0.5 m, with two string ties sewn into a fold at the 0.5 m edges. A gardening wire support sewn into a tuck made in the middle, parallel to the string ties to hold the mesh outwards, and a velcro fastening sewn down each of the 0.75 m edges (figure 3.19 + 3.17). A polyethylene

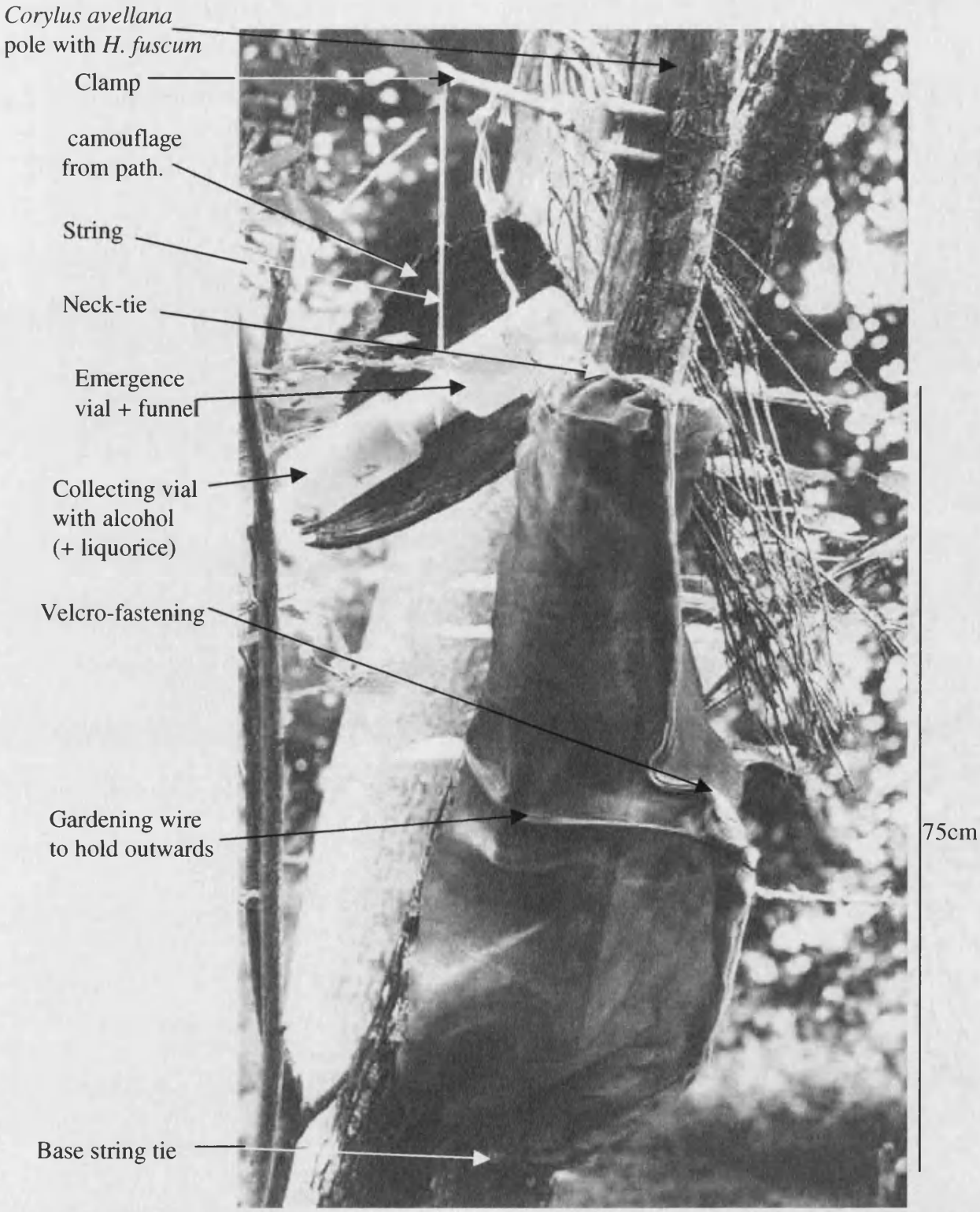


Figure 3.7. One of 10 field traps erected in the study site. The trap consists of the finest plastic gardening netting, small enough to let air through but no insects, sewn with gardening wire supports to hold the netting away from the logs, with velcro fastenings so it can be wrapped around the log of interest and tied and sealed around the top and bottom. The dual assembly collecting vial is for easy collection and processing of results at monthly intervals. 70% alcohol is placed to a depth of 3 cm in each vial to preserve all the incumbents.

funnel was glued through a hole melted through the mesh so that the wide end of the funnel was flush with the mesh and the narrow end pushed through to the outside.

3.3.7.2 Erecting traps over small hazel poles

Each trap was wrapped around a chosen standing or horizontal hazel pole and the Velcro fastened carefully so as not to allow any spaces through which invertebrates might gain entry or escape. The ends were then sealed off using the string ties. The gardening wire was adjusted if necessary to ensure that the edges of the netting were held outwards away from the bark. A tie was used to support the funnel to ensure it was held as the uppermost part of the trap. Finally, a small 50 cm^3 plastic collecting bottle which had a hole melted into its side so as to fit tightly to the opening of the trap's funnel, was filled with 15 cm^3 of 75 % alcohol, and attached to the trap.

3.3.7.2 Siting traps in the study site

Ten traps were fastened around hazel logs on which cecid larvae and fungi had been found. The Velcro fastenings were carefully sealed leaving no holes through which insects might escape, whilst the string ties, and the tight buckling of mesh which occurred as they were tightened around the log, sealed any undulations in the bark surface to prevent invertebrates from crawling out. The collecting bottles were held out so that they formed the highest point of the trap with aid of a boss, clamp and string. Each month, bottles were removed and replaced, and invertebrate samples brought back to the university for sorting and recording taxonomic groups, orders and families. Four of the traps also contained approximately 2 g of liquorice root shavings placed into the alcohol, within collecting bottles to test the effect of liquorice odour on the ability to trap insects. The liquorice investigation was based on a laboratory observation (Scale 7) that melanised and PSP regions of *Hypoxylon fuscum* cultures smelt strongly of liquorice (see chapter 6).

3.3.8 methods of magnification and measurement of field cecid larvae

Due to the small size of cecid larvae, polarised light (figure 3.3) and scanning electron microscopy were necessary to see cuticle details, which are necessary for identification of genus and species (figure 3.4). Field measurements of larvae and their sub-cortical cavities were made using a field microscope lens with calibrated eyepiece graticule, capable of measuring details *in situ* from 1 cm down to 0.05 mm in size. Measurements larger than 1 cm were made using a flexible meter tape. Photographs were taken with a camera equipped either with an extension-tube or, later on in the project, with a macro-lens.

3.3.9 Temperature measurement methods

Measurements of temperature were made using a double thermocouple probe attached to a digital thermometer, which could display both readings simultaneously to see the difference between ambient and sub-cortical conditions (Unwin 1980). A small hand drill was used to drill holes into the bark and sub-cortical zone, into which one of the probes was inserted. The other probe monitored the ambient temperature just outside the log. Care was taken when inserting the probes not to touch them but to manipulate them with pliers to prevent heating. These measurements were made on hot days in the middle of summer 1998 and also on frosty nights in January 1999 and 2000.

3.3.10 methods of Cecid "jumping" measurement

An unexpected field discovery was made of first-instar *B. fraxinicola* cecid larvae "jumping" out from a vertical hazel pole containing a source habitat's phenomenal population density around a *Hypoxylon fuscum-Hymenochaete corrugata* PSP zone. The colony was situated approximately 1.5m above the leaf litter in a standing coppice to the edge of the study site. The weather had just turned sunny after a period of July rain. Photographs were taken and larvae collected and brought back to the laboratory with some of the overlying bark. An arena was constructed in the laboratory to measure cecid-jumping trajectories. It consisted of the radial markings of a nested quadrat placed below a petroleum jelly coated Petri dish lid - the height of which could be adjusted using string supports to overlying boss and clamps. Two perameters were measured: height jumped and distance jumped. The protocol was to place a cluster of first-instar cecids, removed from a piece of bark freshly removed from the field, into the centre of the nested quadrat arena with a wet fine artist's paint brush. Height measurements were made by lowering the Petri dish lid freshly wiped with a thin layer of petroleum jelly, to a certain height above the cecid cluster. After 15 minutes the number of cecids stuck to the petroleum jelly were counted, removed and a new layer of petroleum jelly applied. A new cluster of cecids was positioned and the lid lowered to a new height. Distance measurements consisted of placing a cluster of cecids into the centre of the arena without the Petri dish lid being present, and counting the numbers of cecids which lay between radial markings of the nested quadrat after two minutes. These were removed with an artist's paint brush so that they could be used in culture experiments (chapters 4 - 6). A fresh untested cluster of cecids from the woodland sample was placed in the arena, and the trial repeated. All jumping trials were conducted with "fresh" woodland specimens and no larvae were tested more than once.

3.3.11 Statistical analyses methods

Student's t-test was used to compare the degree of overlap between normally distributed parametric data sets where each sample size was greater than nine. When the two data sets being compared had sample sizes less than 9, a non-parametric Mann-Whitney test was used instead since normal distribution of data could not be ascertained (Wheater & Cook 2000). In each case, significance was based on a probability of less than 5 % ($P<0.05$) that the results were caused by chance (Cadogan & Sutton 1994) and the tests were carried out using Minitab for Windows software. The Fisher's Exact test was used to determine significance of frequency data in the log piles from which percentage tables were calculated. The Fisher's Exact test can be used instead of Chi-squared test when the expected value is less than 5 (Chi-squared test needs expected values to be greater than 5) (Wheater & Cook 2000). The Fisher's Exact test was carried out using crosstabulation in SPSS for Windows software. Correlation analysis was used to test both strength and significance of relationships between independently associated variables. Correlation strength is measured as a coefficient between 0, (zero strength), and 1, (maximum strength). Correlation significance is measured as significance (at $P\leq0.05$) of relationships between two measured factors. For a correlation to be asterisked in the results tables, it had to have a high strength and also a less than 5 % probability that the scatter of points could have arisen by chance (Wheater & Cook 2000).

3.3.12 Fractal dimensions

Fractal dimensions were calculated by taking the gradient from graphs of nested quadrat data having plotted the log of the number of units counted on the y-axis against the log of the radius of view from the centre of the nested quadrat (Ramsdale & Rayner 1997, Hartley 1999, West & Deering 1995, Schmid 2000).

3.4 Results

3.4.1 (Scale 1) Landscape distribution

Figure 3.8 shows the landscape distribution of populations of logs and standing hazel poles containing metapopulations of fungi with cecids, marked with red dots onto the Ordinance Survey map of the area. Two fractal dimensions, D1 and D2, were calculated as gradients of plots of the logarithm of population numbers (y-axis) against logarithm of radius in which populations were counted from the centre of the study site (x-axis) (figures 3.6 & 3.8). D1 was found to have a fractal dimension of 0.6 for radii between 30 and 150m from the study site centre, whereas D2 was found to have a fractal dimension of 0.3 at radii between 300 and 650m from the study site centre. These fractal dimension differences indicate that the efficiency of space filling of the pattern of fungal-cecid meta populations within the landscape changes with radius of search from the study site.

3.4.2 (Scales 2 and 3) Population and larval distribution - results

Figure 3.6 and 3.9 (1) show the distribution of logs containing cecid larval populations (in red) within log piles. Figure 3.9 (2) shows the mean distribution of cecid larvae in red counted on a log that has had the bark removed. Exponential curves shown at right show how cecid numbers increase as diameters of view increase. Logarithmic plots show straight lines of different fractal dimensions or efficiency of space filling. The fractal dimension is highest for cecids on a log (D=2.36), followed by cecid-fungal colonised logs (D=1.48), to metapopulations in the landscape (D=0.6 and 0.4).

3.4.3 (Scales 4 - 6) Results of investigation of *Corylus avellana* bark layers

Interactions between the field cecids and fungi were directly observable within bark and sub-cortical surfaces just below the bark. Here there was clear evidence for effects of the insects on fungi and for effects of the fungi on insects. Figure 3.10 shows a log's sapwood surface from which bark layers have been removed. There are two interacting species of fungi, *Hypoxylon fuscum* and *Hymenochaete corrugata*. These were identified through production of fruiting bodies and subsequent culturing (see methods of chapter 4). If the culturing produced a demarcation zone of darkly melanised mycelial pseudosclerotial plate, then the two fungi were shown to be of different species. It was on such pseudosclerotial plates that the majority of cecid larvae were found. The same type of interaction zone can be seen in cross-section with bark layers intact. Where there were fungal interfaces producing pigmented areas, I found that deeply pigmented bark layers accommodated a greater proportion of cecid larvae than less pigmented and melanised regions. Figure 3.11 shows the architecture of more places where cecids were found, including the insides of highly fractal, melanised fruiting body structures of the ascomycete *Hypoxylon fuscum*, cavities between medullary rays at the surface of the sapwood and the underside of the lowermost layer of bark, and blisters between the top two layers of bark where *H. fuscum* fruiting bodies have ruptured through. This architecture has not been investigated before as space for accommodation of insect larvae. This, as far as I know, is the first data-supported record of such larval accommodation within the *Hypoxylon sp.* group of Ascomycetes.

Table 3.1 log pile correlation (scale 2)

(n=44)

Correlations (ranked) FIELD LOG PILE DATA

Strength	length(cm)	width(cm)	maxht(cm)	minht(cm)	max vol(m3)	minvol(m3)	av vol(m3)	no logs	max diam(cm)	min daim(cm)	Hf	No fungi
length(cm)	1	**0.3**	**0.26**	0.095	**0.329**	**0.153**	**0.292**	**0.227**	**0.11**	0	0.025	0.036
width(cm)		1	**0.309**	**0.112**	**0.764**	**0.325**	**0.667**	**0.375**	**0.126**	0.023	0.003	0.007
maxht(cm)			1	**0.497**	**0.83**	**0.497**	**0.799**	**0.82**	**0.39**	**0.131**	0.026	0.049
minht(cm)				1	**0.413**	**0.953**	**0.662**	**0.603**	**0.148**	**0.169**	0.067	0
max vol(m3)					1	**0.543**	**0.933**	**0.845**	**0.223**	0.085	0.005	0.047
minvol(m3)						1	**0.786**	**0.67**	0.096	**0.144**	0.027	0.004
av vol(m3)							1	**0.89**	**0.195**	**0.116**	0.012	0.03
no logs		Key:						1	**0.175**	**0.11**	0.011	0.089
max diam(cm)		**bold** = slope > 0.1							1	**0.102**	0.082	0.002
min daim(cm)										1	0.014	0.004
Hf											1	0.003
No fungi												1

Correlation (ranked) FIELD LOG PILE DATA

P. nos	length(cm)	width(cm)	maxht(cm)	minht(cm)	max vol(m3)	minvol(m3)	av vol(m3)	no logs	max diam(cm)	min daim(cm)	Hf	No fungi
length(cm)	Zero	**0.007**	**0.013**	0.15	**0.008**	0.09	**0.014**	**0.025**	0.12	0.9	0.46	0.4
width(cm)		Zero	**0.006**	0.118	**0.0001**	**0.009**	**0.0001**	**0.002**	0.097	0.48	0.8	0.7
maxht(cm)			Zero	**0.0001**	**0.0001**	**0.001**	**0.0001**	**0.0001**	**0.001**	0.09	0.5	0.3
minht(cm)				Zero	**0.002**	**0.0001**	**0.0001**	**0.0001**	0.07	**0.05**	0.2	0.95
max vol(m3)					Zero	**0.0001**	**0.0001**	**0.0001**	**0.035**	0.2	0.7	0.3
minvol(m3)						Zero	**0.0001**	**0.0001**	0.18	0.1	0.5	0.48
av vol(m3)							Zero	**0.0001**	0.052	0.14	0.6	0.19
no logs		Key:						Zero	0.053	0.13	0.6	0.8
max diam(cm)		**bold** = P <0.05							Zero	0.14	0.18	0.86
min daim(cm)										Zero	0.6	0.77
Hf											Zero	0.8
No fungi												Zero

Table 3.1 shows correlation strengths and significances made on data from 44 log piles measured in the study site. No significant relationships could be found between the fungal community diversity and the frequency of occurrence of *Hypoxylon fuscum,* or of the size of log piles. The most significant relations were between log pile dimensions such as height and overall log pile volume, number of logs, and size of largest logs in pile with overall pile dimensions. Table 3.2 shows mean data from the 44 log piles.

TABLE 3.2 Means of 44 log pile measurements

Log pile measurement	Mean value (n=44)
Pile length (cm)	219.0
Pile width (cm)	163.9
Height of pile (cm)	30.3
Volume of pile (m3)	1.60
Number of logs in pile	42.4
Diameter largest log in pile (cm)	12.9
Diameter minimum log in pile (cm)	3.3
no logs / pile with *H. fuscum*	7.08
estimated no. fungal species / pile	5.2

Table 3.3 shows macro-fungal biodiversity and abundance data for the field site cumulated from the rapid macro-fungal survey technique (chapter 2). In four study site plots, 53 species of fungus were found growing on wood of 5 species of tree: ash, hazel, sycamore, blackthorn and hawthorn and associated ground vegetation.

TABLE 3.3 MACRO-FUNGAL BIODIVERSITY OF STUDY SITE 1995-1996

Fungal Species	rank	Frequency	cecids	cultured with cecids
Vuilleminia commedens	1	22	*	+
Hypoxylon fuscum	2	19	*	+
Hymenochaete corrugata	3	15	*	+
Hypoxylon numularium	4	9	*	+
Mycelial fans	5	8		
Mycelial chord	6	8		
Stereum hirsutum	7	6		+
Lichens	8	5		
white cup ascomycete	9	5		
Stereum gausipatum	10	5		+
white slime mould	11	5		
Diatripella disciformis	12	4	*	
Bisporella citrina	13	3		
Coriolus versicolor	14	3	*	+
Daldinia concentrica	15	3		
Exidia glandulosa	16	3		
Mycena sp.	17	3		
Hyphodontia sambuci	18	3		
Merasmius sp.	19	2		
Corticium evolvens	20	2	*	+
Phragmidum violacum	21	2		
Polyparacea sp.	22	2		
Tremella messenterica	23	2		
Stereum rugosum	24	2		+
Phallus impudicus	25	2		
Hypnochinium vellerum	26	2		
Periophora lycii	27	2		
Hypoxylon fragiformi	28	2		+
Incrustiaporia semelipleata	29	1		
Collybia sp.	30	1		
Periophora incarnata	31	1		
Coriolus albietinus	32	1		
Coriolus albidus	33	1		
Coniophora puteana	34	1		
Trichoderma sp.	35	1		
Coprinus radians	36	1		
Diatripella stigma	37	1		
Xylaria polymorpha	38	1		
Xylaria hypoxylon	39	1		
Merullus tremellosis	40	1		
Auricularia judae	41	1		
yellow slime mould	42	1		
Bulgaria inquinans	43	1		
Exidia glandulosa	44	1		
Merasmius sp.	45	1		
Calocera cornea	46	1		
Ascocoryne sarcoides	47	1		
Coprinus sp.	48	1		
Leacocarpus fragilis	49	1		
Dacrymyces stillatus	50	1		
Badhamia cuticularis	51	1		
Crepidotus sp.	52	1		
Phanaerochaete velutina	53	1		+
TOTALS	**53**	**169**	**7**	**11**

MARGALEF BIODIVERSITY INDEX = 53-1 / Ln 169

D.Mg = 52 / 5.129

D.Mg = 10.14

Table 3.4 Individual log correlation (scales 3 - 5) (n=30)

Key: **bold** = slope > 0.1

Correlation FIELD LOG DATA

Strength	no.fungalspecies	no.Insect species	no moss species	length log	diam log
no.fungalSP	1	**0.399**	**0.555**	0.006	0.023
no.InsectSP		1	**0.27**	**0.21**	0.045
no mossSP			1	0.007	0.012
length log				1	**0.135**
diam log					1

Key: **bold** = P <0.05

Correlation FIELD LOG DATA

P nos.	no.fungal species	no.Insect species	no moss species	length log	diam log
no.fungalSP	Zero	**0.002**	**0.0001**	0.7	0.45
no.InsectSP		Zero	**0.01**	**0.032**	0.345
no mossSP			Zero	0.68	0.6
length log				Zero	0.065
diam log					Zero

Twenty-six (half) of the fungus species were recorded only once. The most commonly encountered fungus was *Vuilleminia comedens* that was found at 21 locations, followed by 19 locations for *Hypoxlyon fuscum*. A Margalef diversity index of 10.14 is shown below table 3.3. Asterisks (*) indicate fungi with which cecids were associated in log piles and standing hazel poles. Crosses (+) indicate fungi that were isolated for laboratory studies on which cecids were successfully cultured in laboratory conditions (chapter 4).

The frequencies of finding cecids under bark with a species of fungus are shown in figure 3.12. In the study site area, 15 cecid larval colonies of species *Brittenia fraxinicola* were found on *Hypoxylon fuscum*, 10 on *Vuilleminia commedens*, eight on *Diatripella disciformis*, six on *Mycoacia uda*, and between three and one on each of *Hymenochaete corrugata*, *Hypoxylon nummularium*, *Stereum hirsutum*, *Coriolus versicolor*, *Corticium evolvens*, *Hypoxylon fragiforme*, *Piptoporus betulinus*, *Heterobasidion annosum*, *Puccinia sp.* and *Libertella sp.* All cecid colonies were found on wood itself colonised with more than one species of fungus, which means that there was some degree of overlap regarding the above data. No cecid colonies were found under bark of logs colonised by only one species of fungus. Cecids found in this situation were occasional and usually solitary.

Table 3.4 shows correlation between data collected on 30 single logs. The most significant relations were between the number of moss, fungal and insect species ($P<0.01$). However, only the number of insect species was correlated significantly with length of logs. The diameter of a log does not relate significantly to diversity of fauna and flora found on it. Table 3.5 shows mean data from 30 logs.

TABLE 3.5 Mean data per log (n=30)

Measurement	Mean
no. fungal species	3.9
no. insect species	3.5
no. moss species	1.5
length of log (cm)	158.2
diameter of log (cm)	8.2

Figure 3.13 shows how the number of exit holes made by a particular species of wood boring beetle increases with pigmentation of the log. Table 3.6 shows percentage overlap between measured variables and also Fisher's Exact test performed on raw frequency data for Chi-squared associations when the expected is less than 5 (Wheater & Cook 2000, Bailey 1981). The most significant results are shown next to an asterisk and the least significant with round dots. The values in the percentage table only show overlap between the variable across the top of the table (horizontal axis) with those on the vertical axis. For example, 40 % of logs positioned in the centre of piles (horizontal axis) had Collembola in their sub-cortical zones (vertical axis), whereas just 18 % of logs in total with Collembola on them (horizontal axis) occurred at the centre of log piles (vertical axis). In decreasing order of % overlap pairs, what this table shows is that 100 % of logs in the centre of log piles possessed visible evidence of slime mould "grazing" whereas only 42 % of logs with slime mould evidence occurred in the centre of log piles. 95 % of logs with cecids had pseudosclerotial plate (PSP) and 85 % of logs with PSP had cecids. 94 % of logs with snails had PSP zones, whilst 85 % of logs with PSP had snails. 92 % of logs with slime mould had PSP and only 55 % of logs with PSP had slime mould evidence on them. 91 % of logs with Collembolans and 83 % of logs with cecid larvae also had bark cracks whereas of logs with bark cracks, 79 % of them contained cecid larvae and only 52 % had any visible Collembola. Of logs with cracked bark, 89 % were located near the outer extremity of log piles and 86 % had

Table 3.6 Fishers' Exact test for individual logs (n=30) (scales 3-5)

% top with bottom	brk.cracks	psp	edge pile	centre pile	snails	slime mould	cecids	collembola	Moistbark
brk.cracks?		* 83	* 81	40	* 82	* 75	* 83	* 91	* 88
psp?	* 86		76	* 80	* 94	* 92	* 95	* 82	* 81
edge pile?	* 89	* 80		------	* 76	58	* 83	* 82	* 87
centre pile?	• 11	20	------		24	42	17	18	• 13
snails	74	* 85	62	80		* 92	* 78	63	63
slime mould	– 47	55	33	* 100	65		– 56	– 55	– 56
cecids?	79	* 85	71	60	* 83	* 83		* 82	75
collembola?	– 52	– 45	42	40	41	– 50	– 50		– 56
Moistbark?	74	65	67	40	59	75	67	* 82	

% = * = high % overlap ≥ 75
• = low % overlap ≤ 15
– = half overlap % ≅ 50

Fisher's exact P numbers	brk.cracks	psp	edge pile	centre pile	snails	slime mould	cecids	collembola	Moistbark
brk.cracks?	zero	0.293	0.101	* 0.066	0.188	1 •	* 0.066	* 0.09	* 0.05
psp?		zero	1 •	1 •	0.1	0.17	** 0.002	0.661	0.63
edge pile?			zero	----	0.628	0.12	0.597	1 •	0.321
centre pile?				zero	0.628	0.12	0.597	1 •	0.321
snails					zero	0.14	0.58	1 •	1 •
slime mould						zero	0.378	0.695	0.411
cecids?							zero	0.407	0.673
collembola?								zero	0.208
Moistbark?									zero

Significance

** = P < 0.05
* = P < 0.1
• = P = 1

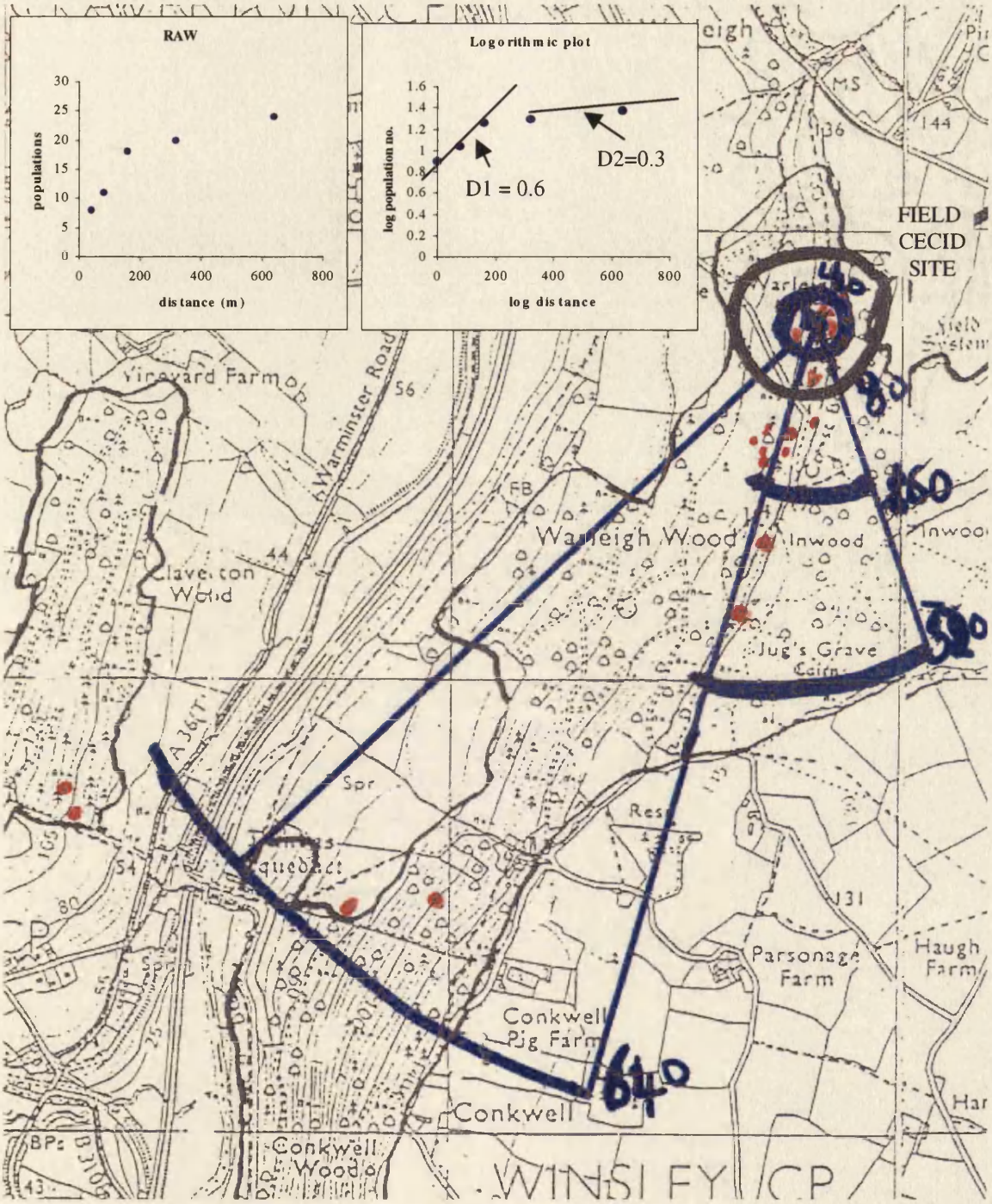


Figure 3.8 Ordinance survey map of field site on ridge overlooking the river Avon valley as it winds towards Bath. Red dots indicate field cecid populations of *Brittenia fraxinicola* all found within dead logs of hazel coppice. Blue lines show the extended nested quadrat through the Avon landscape with distances from centre of field study site in metres. Two graphs show no. populations within radius of view (RAW), and fractal dimensions (Logorithmic plot) at the scale of populations of *Corylus avellana* with *H. fuscum* and *B. fraxinicola*.

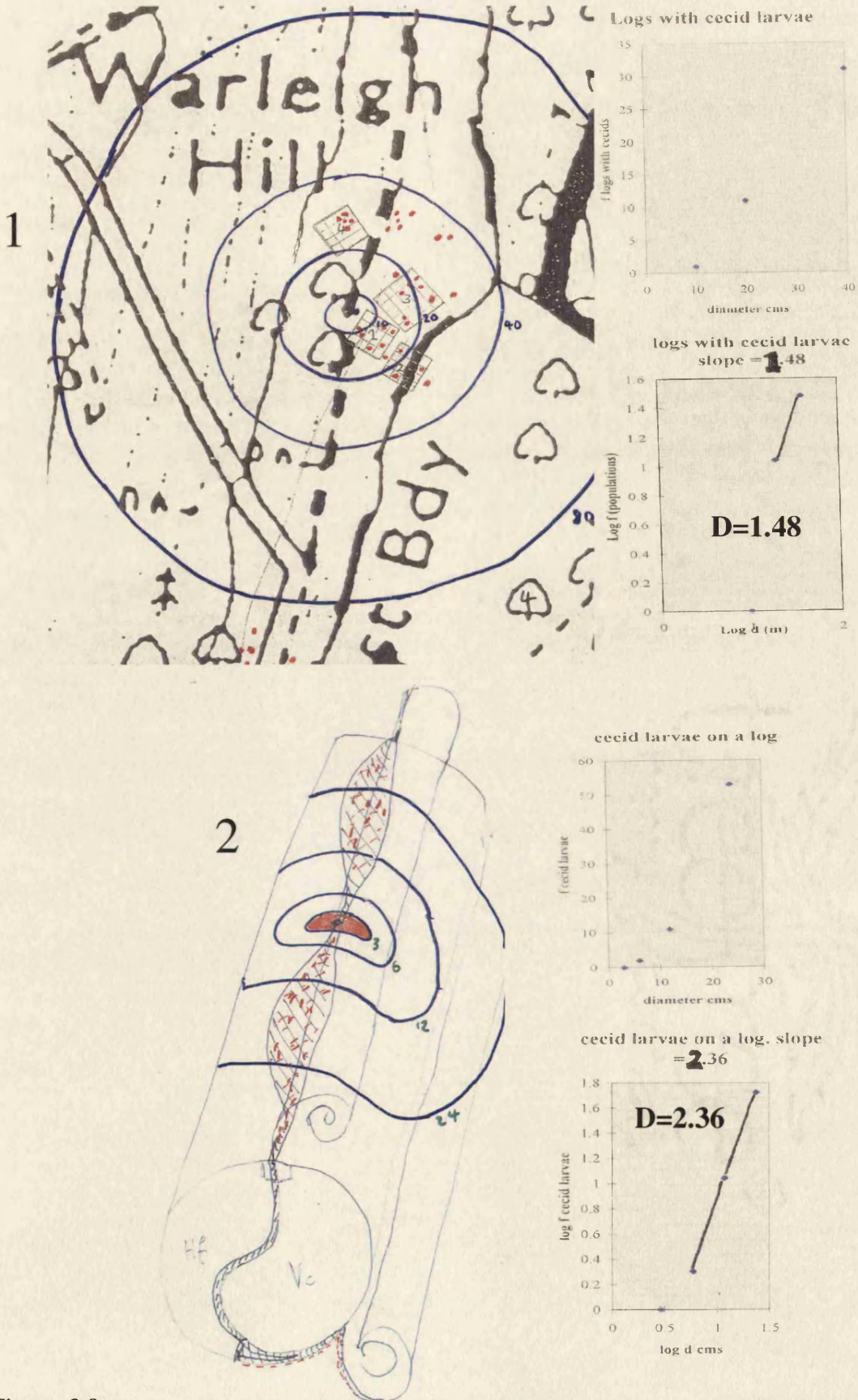


Figure 3.9 (1) Nested quadratting of logs containing resident communities of cecids and fungi in study site. (2) cecids on a log inhabited by fungi. The fractal dimension of each scale of viewing cecids, fungi and wood is shown as **D** - the slope of the logorithmic plot of population number per radius of view.

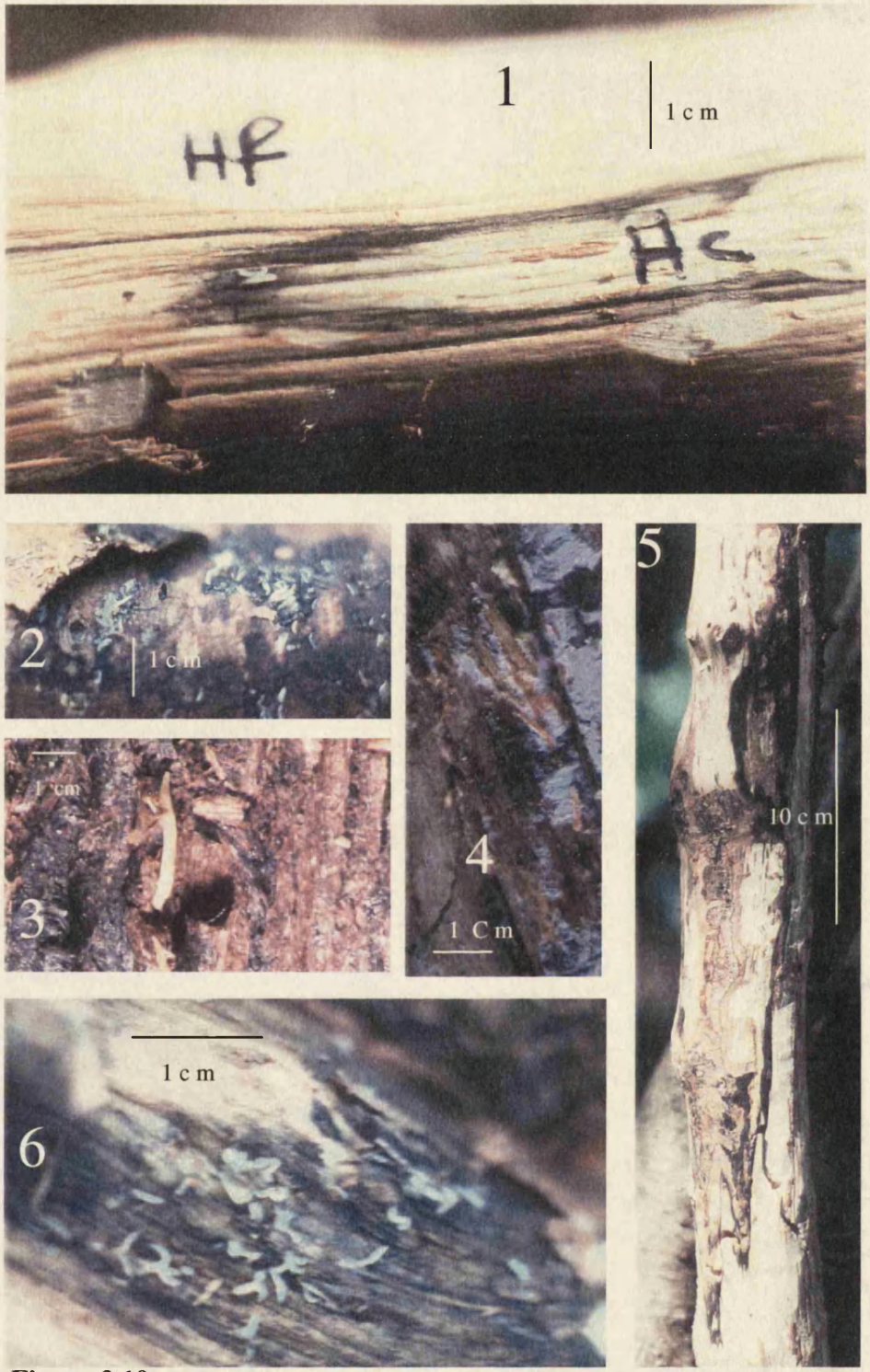


Figure 3.10 The finding of cecids associated with PSP zones; 1; two cecids are visible on the PSP zone between *Hypoxylon fuscum* and *Hymenochaete corrugata* on a hazel log. 2; where the PSP is diffuse between bark layers, the cecids possess a more heterogeneous distribution, 3; cavities seem more numerous and sizes appear to be greater in PSP zones, here to the left of a hemipupa, rather than in non PSP zones, where cavities seem less apparent. 4; A fungus known to parasitise other fungi *Mycoacea uda,* is visible on the sap wood surface surrounding cecid hemipupae which have died and turned brown. 5; PSP is easy to come across in any hazel logs large enough to accommodate 2 or more macro-fungal species. 6: A range of different sizes of cecid emerge from cavities in PSP wood.

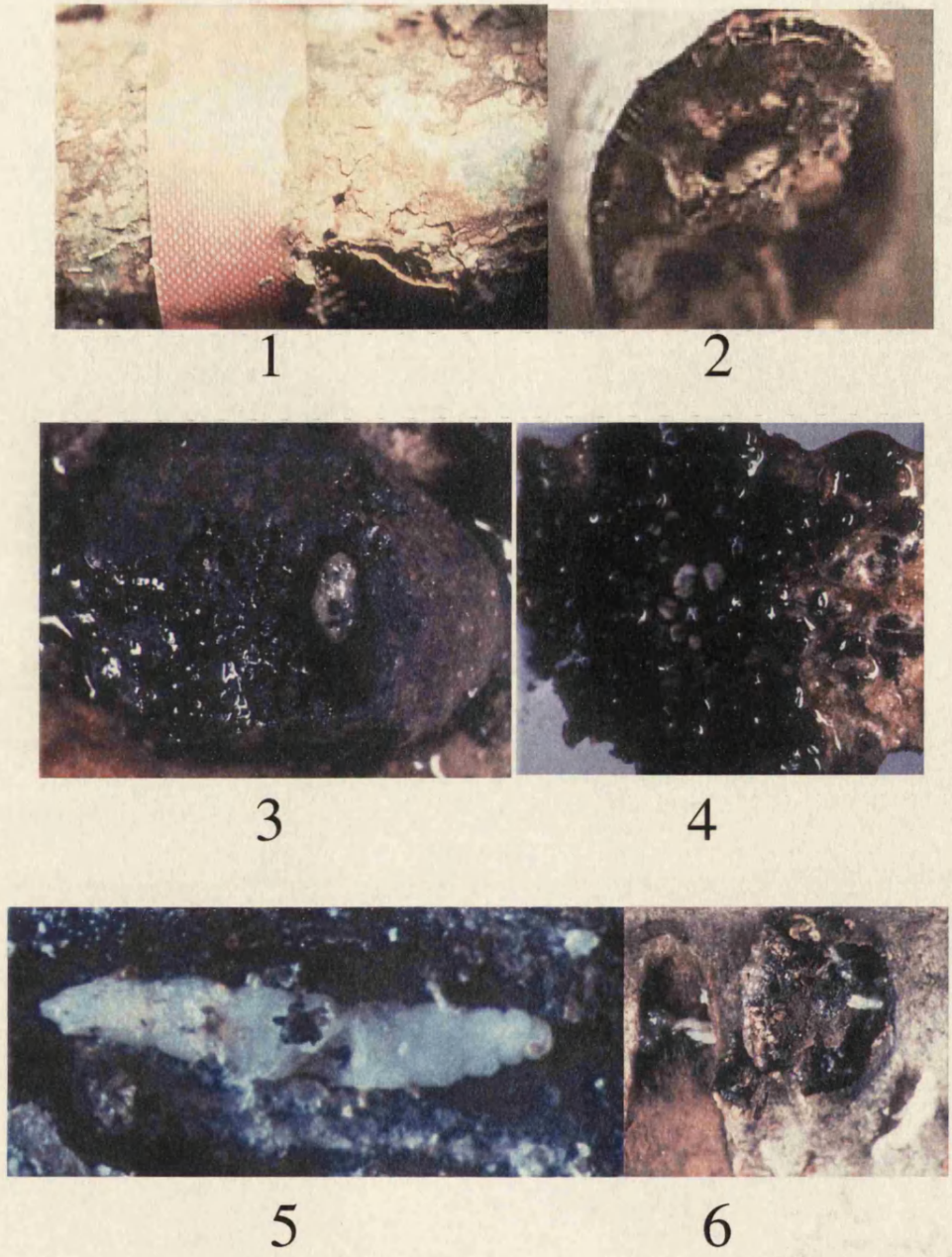


Figure 3.11 The relations of cecids in a dynamic world. (1) shows observation of temporal spread of fungal fruiting hymenium over field site flagging tape. The fungus is *Hymenohaete corrugata*. (2) shows the result of cavitation processes within decomposing wood. (3) shows a large dipteran larva inhabiting cavities Within the fruiting bodies of *Hypoxylon fuscum*. (4) shows the cavitation within the same *Hypoxylon fuscum* to be fractal in geometry. (5) shows a large cecid larval hemipupa inhabiting a fissure in pseudosclerotial plate. (6) shows smaller larvae inhabiting cavities within *H. fuscum* fruiting bodies.

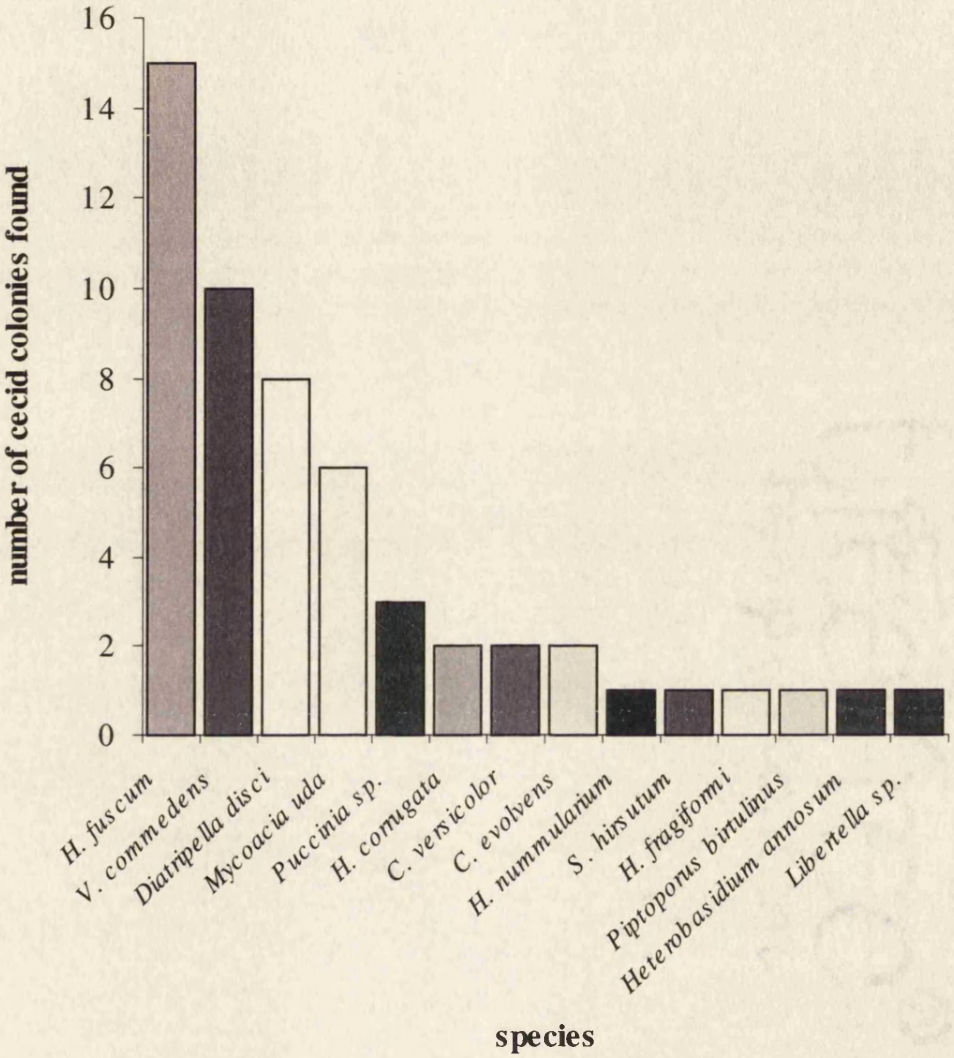


Figure 3.12 Frequency of finding cecids under bark of logs colonised by particular wood decay fungi in the cecid field site near Bath.

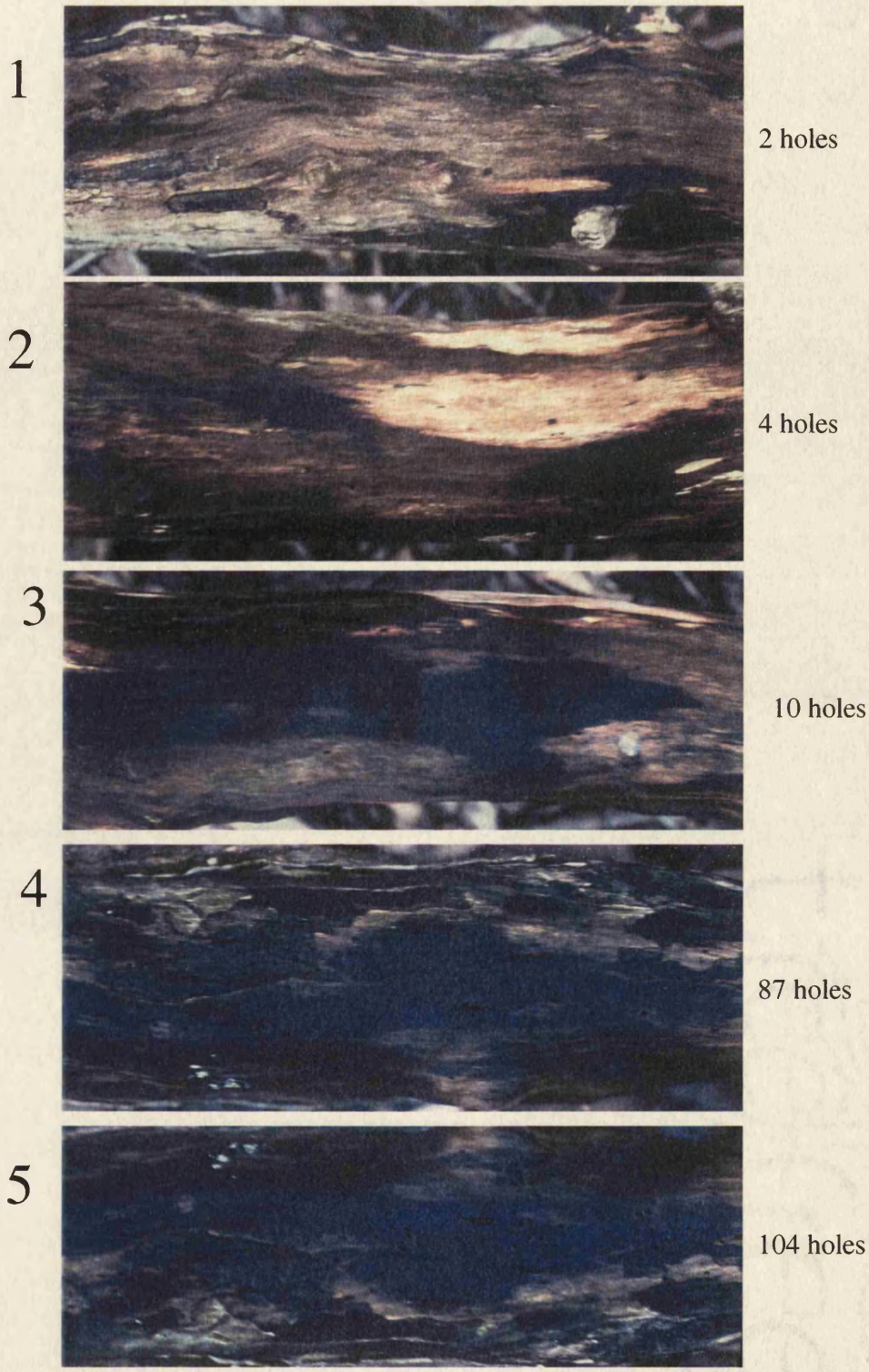


Figure 3.13 Photographs taken from a standard height above regions of the same log on the ground. Each image represents a quadrat of that log where bark has fallen away leaving blackened PSP covered sap-wood surface which insulates fungal mycelia within. What is of interest is to compare the number of insect beetle holes between quadrats on the same log, with different amounts of PSP pigmentation. Quadrats 1 and 2 have less PSP and fewest beelte holes. 3 has some holes – mainly associated with PSP whilst 4 and 5 show a large number of easily visible insect holes widely spread over the sap wood surface. It is likely that the same fungal individuals extend in interwoven decay columns throughout in each image, and that the coleoptera belong to the same population wood boring beetles.

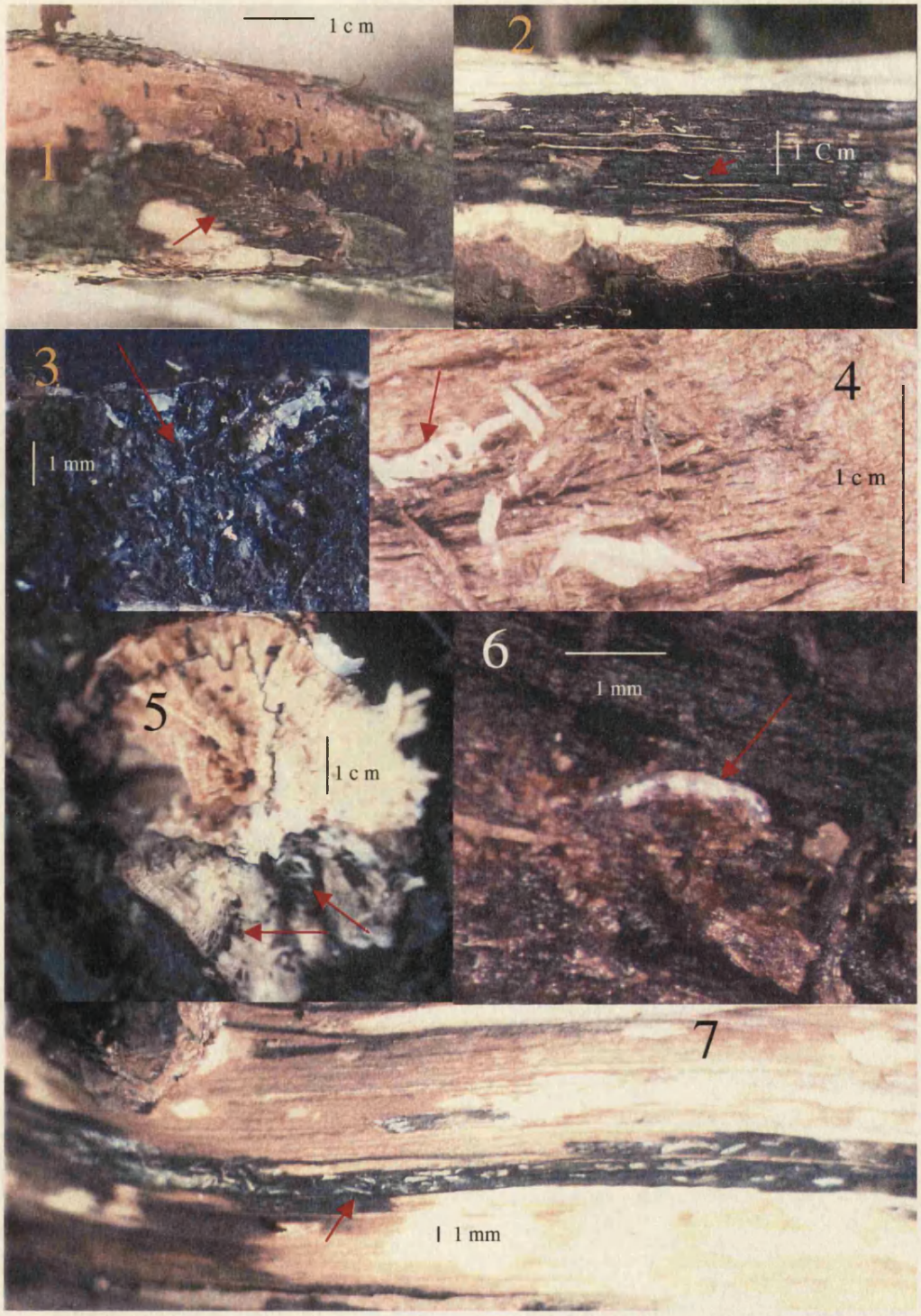


Figure 3.14 1; a hazel log with a fruiting body of the resident fungus *V. commedens* is cut to locate positions of cecid larvae in sub-cortical zone (larvae arrowed). 2; cecid larvae (arrowed) are visible negotiating inter-medullary ray channels on the surface of PSP covered sao-wood. 3; cecid-created channels through softer areas of frass- filled PSP zone can be seen (arrowed). 4; a range of differently sized cecids (arrowed) are visible inside bark cavities in the bark's underside. 5; A standing hazel pole, snapped off near the ground, reveals large cavities stretching up from root base which are lined in PSP. 6; A cecid larva (arrowed) found in a small cavity between sap wood (brown) and PSP zone (black). 7 ; A large population of cecids (arrowed) found running down a PSP zone between the fungi *H. fuscum* and *V. commedens.*

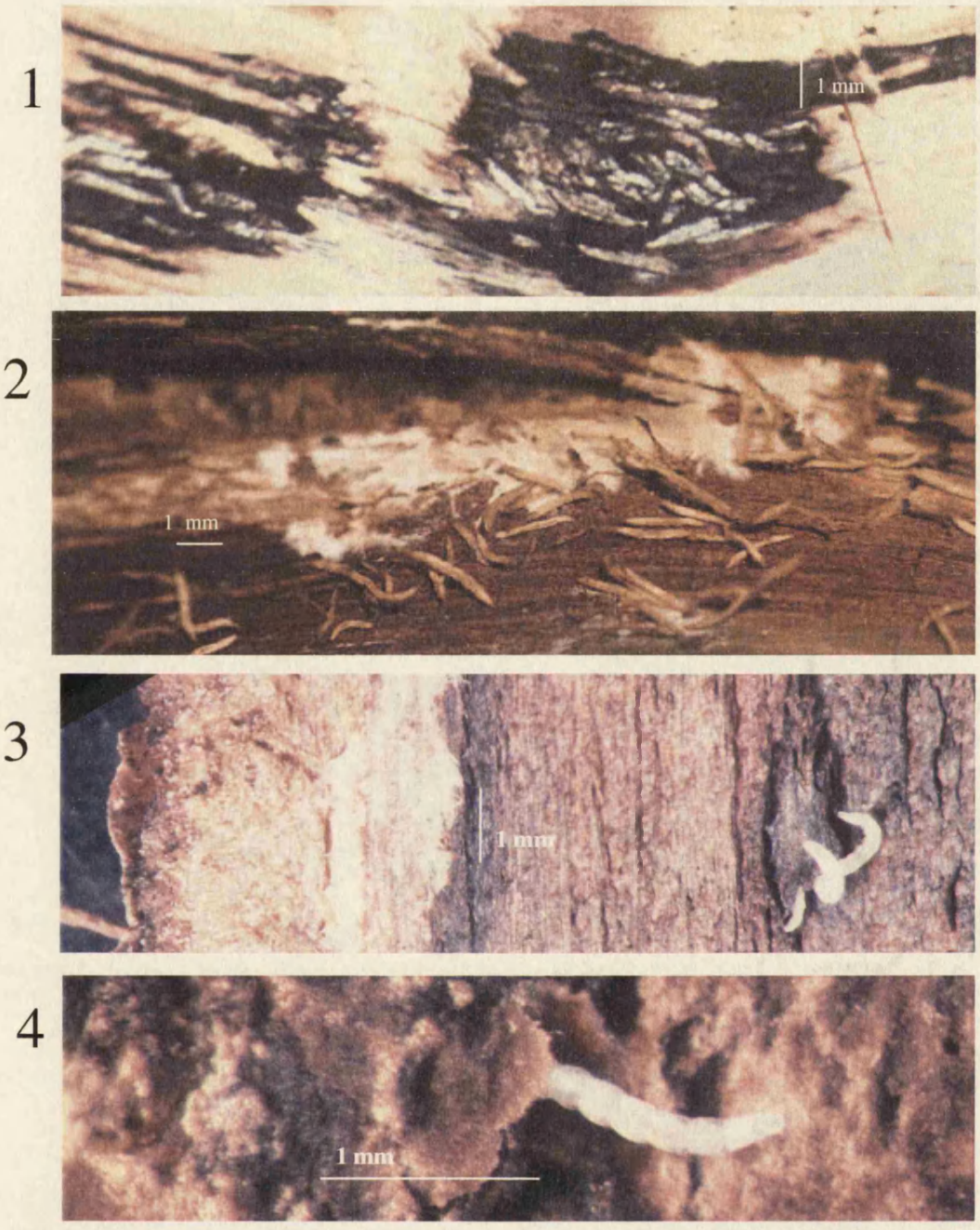


Figure 3.15 (1) shows a sapwood surface view of knot branch point under which a narrow saucer-like cavity accommodates a swirling formation of *B. fraxinicola* cecid larvae. (2) shows a number of hemipupae of the field cecid lying next to the margin of a sub-cortical basidiomycete fungal mycelium. (3) shows two young *B. fraxinicola* disturbed in a cavity a few mm below the top of the sap-wood bark interface. (4) shows a *B. fraxinicola* larva within a sub-cortical resin-like exudate found sub-cortically and observed often with the fruiting body pustules of the ascomycete fungus *Hypoxylon fuscum.*

PSP zones. Of logs with moist sub-cortical zones on a hot mid-summer's day, 88 % had cracked bark, 81 % had PSP zones and 87 % were located near the edge of the pile. The Fisher's Exact test results are shown as p-numbers in the significance portion of table 3.6. The most and only highly significant relation is between logs with cecids and logs with PSP ($P < 0.005$). This is followed from higher towards lower significance by the relations between moist bark and cracked bark, logs with cecids and cracked bark, and logs with Collembola and cracked bark. To see the cross-tabulations of frequency data from SPSS for Windows software, from which table 3.6 was derived, please see Appendix 4.

3.4.4 Field photography results

Figure 3.14 shows locations of *Brittenia fraxinicola* cecid larvae within sub-cortical and pseudosclerotial plate zones of *Corylus avellana*. Figure 3.15 shows other positions of *B. fraxinicola in situ* as described in the figure text.

3.4.5 Ambient and sub-cortical temperature results

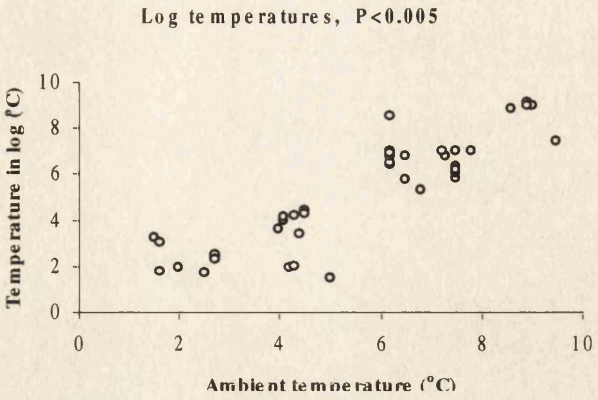
The temperature heterogeneity throughout the forest was particularly noticeable on sunny days and also whenever working in the study site, as I was accustomed to do in summer, with bare feet to avoid damaging underlying mycelia. For example, under the sub-cortical zone of one large piece of woody debris on the forest floor in direct June sunlight 1997, the inner temperature on the surface of sapwood under thick and blackened bark with PSP was 26 °C whereas the ambient temperature was only 22 °C in direct sunlight and a few cm along the log in the shade, the temperature was only 19 °C. Data from measurements of the spatio-temporal temperature heterogeneity of sub-cortical zones are shown in figures 3.16 and 3.17. Figure 3.16 shows, at the top, the only significant relation between the ambient and sub-cortical temperatures ($P<0.005$). The two other graphs on figure 3.16 show ΔT vertically.

ΔT = temperature of sub-cortical layers - ambient temperate (°C)

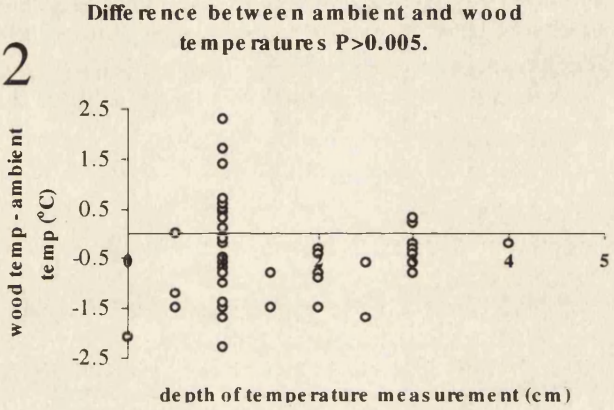
ΔT (+ve) indicates that sub-cortical zones were warmer than ambient temperatures.
ΔT (-ve) indicates that sub-cortical zones were colder than ambient temperatures.

As can be seen in the middle graph in figure 3.16, which shows the variation of ΔT with depth of measurement, there seems a general trend for ΔT to rise from negative towards positive with depth of measurement despite the lack of significance for this correlation ($P >0.05$). Most readings were taken at a depth of 1 cm and it is consequently here that most variance is expressed in ΔT data with equal proportion of ΔT +ve and -ve. Excluding data from 1 cm depth reveals what could be a significant trend towards sub-cortical zones that approach the same temperature as ambient conditions ($\Delta T=0$), but generally sub-cortical zones were between 0.5 and 1.5 °C cooler than ambient temperatures. The bottom graph of ΔT shows the temperature difference between ambient and sub-cortical zone. Here ΔT varied with date of measurement from early January 2000 to April. The times of frosts are shown because it was only during rare below freezing conditions that ΔT became positive. The red line plots a mean "moving average" between every successive pair of data points. To the right are shown correlations of the same data, the only significant relation being between ambient and sub-cortical temperatures. Interestingly, the next near-significant result ($P=0.07$) is between ΔT and ambient temperature whereas the relation between ΔT and sub-cortical temperature is clearly not significant ($P=0.9$).

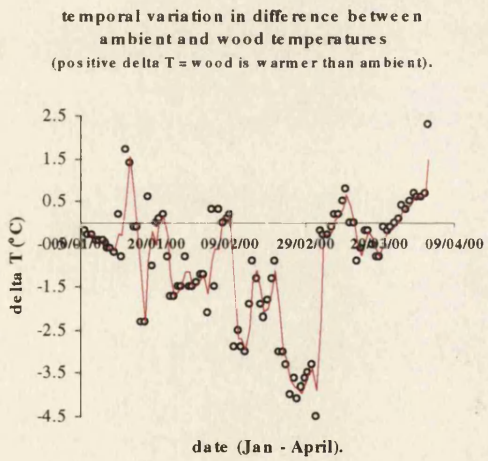
1



2



3



4

Correlation TEMPERATURES				
Strength	T1 (°C)	T2 (°C)	depth	delta T (°C)
T1 (°C)	1	**0.8**	0.023	0
T2 (°C)		1	0.02	0.057
depth			1	0.002
delta T (°C)				1

Correlation TEMPERATURES				
P. nos	T1 (°C)	T2 (°C)	depth	delta T (°C)
T1 (°C)	Zero	**0.0001**	0.28	0.9
T2 (°C)		Zero	0.3	0.07
depth			Zero	0.778
delta T (°C)				Zero

Figure 3.16 (4) shows correlation between habitat temperature (T1) and sub-cortical zone temperature (T2) of decaying logs, plotted at (1) with the difference between the two (delta T) over time (3), and with different depths below bark surface (2). Simultaneous measurements of two temperatures were made using a double thermocouple digital thermometer.

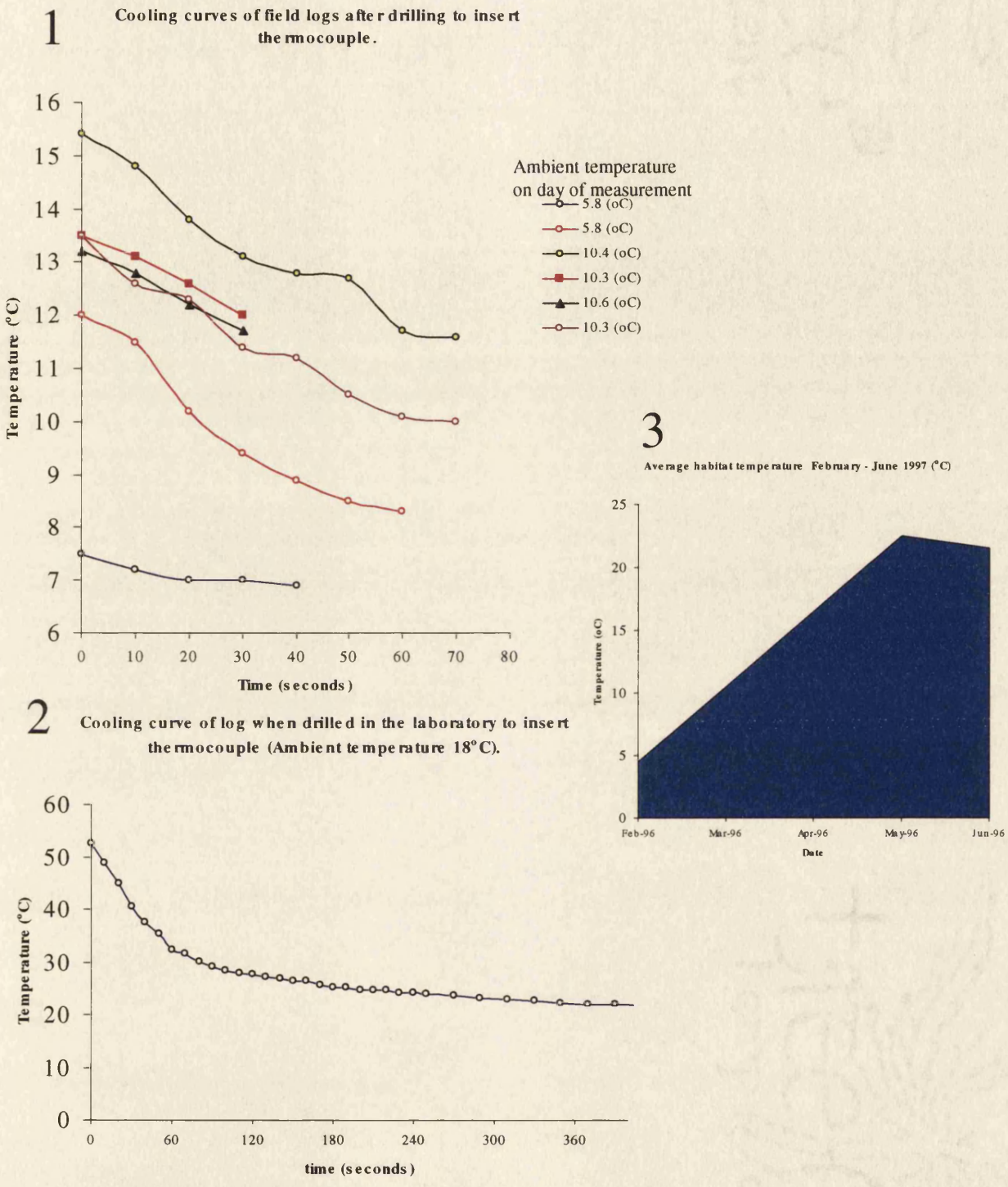


Figure 3.17 Sub-cortical temperature measurements taken after holes were drilled with a 1.5mm drill bit attached to a hand-operated drill, and the thermocouple inserted with a pair of forceps. (1) shows the cooling curves of logs in the field site. (2) shows cooling curve of log in the laboratory, (3) shows average ambient daytime field site temperature Spring 1997.

Figure 3.17 shows how drilling holes in the sub-cortical zone caused heating, which had to be accounted for by leaving round tooth-picks in the pre-drilled holes until the date of measurement (to see pre-drilled holes with picks in position on logs during a winter's field visit, please load the moving image CD rom in back cover of thesis and click on habitat). Very approximate habitat temperatures for early 1997 are shown at middle right in the figure.

3.4.6 Field trap results

Figure 3.7 shows one of the 10 erected field traps with sampling-bottle awaiting collection. An assortment of insect taxa trapped are shown in figure 3.18. As described in the figure text, these included beetles (Coleoptera), Sciarids (Diptera, Sciaridae), parasitic wasps (Hymenoptera), Phorids (Diptera, Phoridae), Mycetophilids (Diptera, Mycetophilidae), micro-moths (Lepidoptera). Although some cecidomyiid adults were indeed captured (Diptera, Cecidomyiidae), none were *Brittenia fraxinicola* and none were paedogenetic genera. Wyatt thought that these cecids were most likely to be predatory in their larval forms (Wyatt 2000). Many orders of insects and invertebrates inhabiting hazel poles were successfully captured. A complete break down of raw trap data into the various identified groups of insect and other invertebrate orders and genera is presented as Appendix 5. Diversity and abundance, as split into families and orders, are shown in figure 3.19. Adult cecid flies were some of the rarer insects with only 40 caught in total and an average of 3.5 per month for all 10 traps. It is worth noting that **all these insects and invertebrates have emerged from an estimated volume of only 0.025 m^3 of wood**.

Figure 3.20 shows the mean catch of each type of invertebrate for all 10 traps over the period February 1998 to August 1999. Data for different Diptera are shown in red lines on the top three graphs (1-3). Graph 1 for cecid adult emergence is uppermost, parasitic wasps in crosses with dashed line (6), and Phoridae (4). The most a-synchronic curve, as compared to the phenology of Diptera and Coleoptera, is shown for earwig populations (5). The top graph (1) of figure 3.20 shows cecid catch per month per trap reaching a highest peak in spring 1998 and then gradually declining to a stable catch of 0.2. This was very similar to catches for Sciaridae but not Mycetophilidae. Generally orders and families identified possessed three major periods of adult emergence. These were early spring, mid to late summer and then autumn. In between such peaks were rapid reductions in adult emergence, for example mid May 1998 (except earwigs), mid October 1998 (including earwigs), January 1999 (all groups), mid April 1999 (except parasitic wasps, beetles, spiders and weevles). Table 3.7 shows correlations of insect trap data for each invertebrate group, using raw data for all 10 traps (Appendix 5). The steeper slopes and greater significances are asterisked. Cecid adult emergence is very closely correlated with that of Sciaridae but not of Mycetophilidae. Emergence of Mycetophilidae and Sciaridae are, however, significantly correlated ($P<0.05$). Cecid adult emergence is also closely tied to that of Phoridae, earwigs and aphids, but not to parasitic wasps, beetles or any other predators ($P>0.05$).

Figure 3.21 shows cecid adult emergence (red columns) over time as compared to all non-cecid catches (light blue columns). The lower graph (2) groups cecid and sciarid flies together (red columns) as compared to all other invertebrate catch (blue columns). The top graph (1) shows cecids only, as compared to all other catches.

Figure 3.22 shows invertebrate catches according to orientation of logs on which traps were erected - either in a standing coppice or in log piles, vertical or horizontal. Using the Mann-Whitney test for non-parametric

Figure 3.18 Examples of trapped insects; divisions on the photographs are in (mm). At top (1), (2), (3), (4), and (5) are imago cecid species caught (all non paedogenetic - Wyatt 2000), (6), (7), (8) (9)(10) are sciarid and mycetophilid diptera. (12) are small beeltes. (13) a large dipteran whereas (14) is a large parasitic wasp, (15) a larger beetle, (16) a smaller parasitic wasp. (17) is a tiny member of wasp genus *Mymar* sp. one of the smallest parasitic genera . (18) is a larger parasitic wasp.

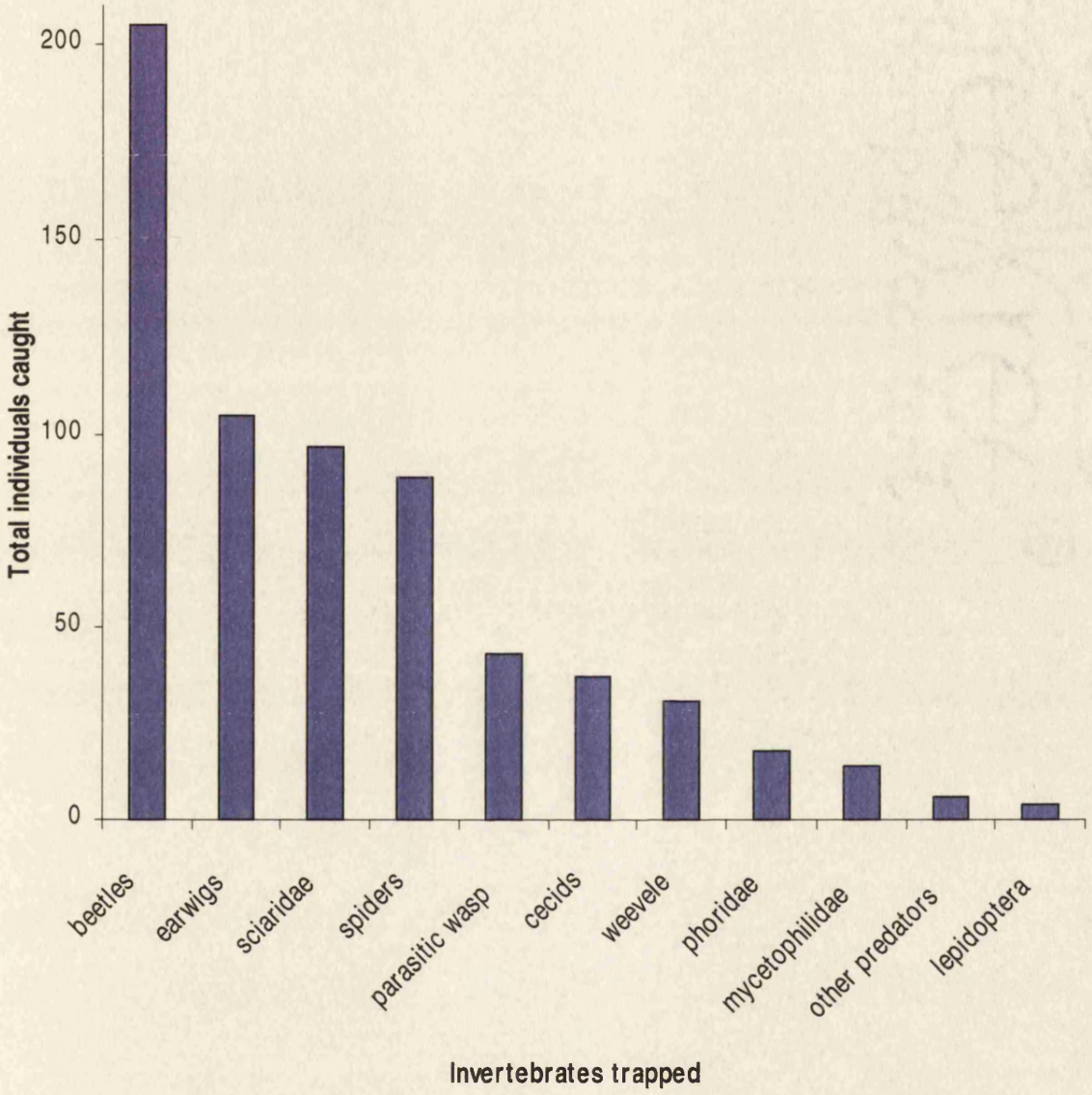


Figure 3.19 Diversity and abundance of insects and invertebrate trapped in 10 emergence traps sited in the study site between January 1998 and August 1999. All the organisms emerged from a total volume of approximately 0.025 m^3 *Corylus avellana* wood.

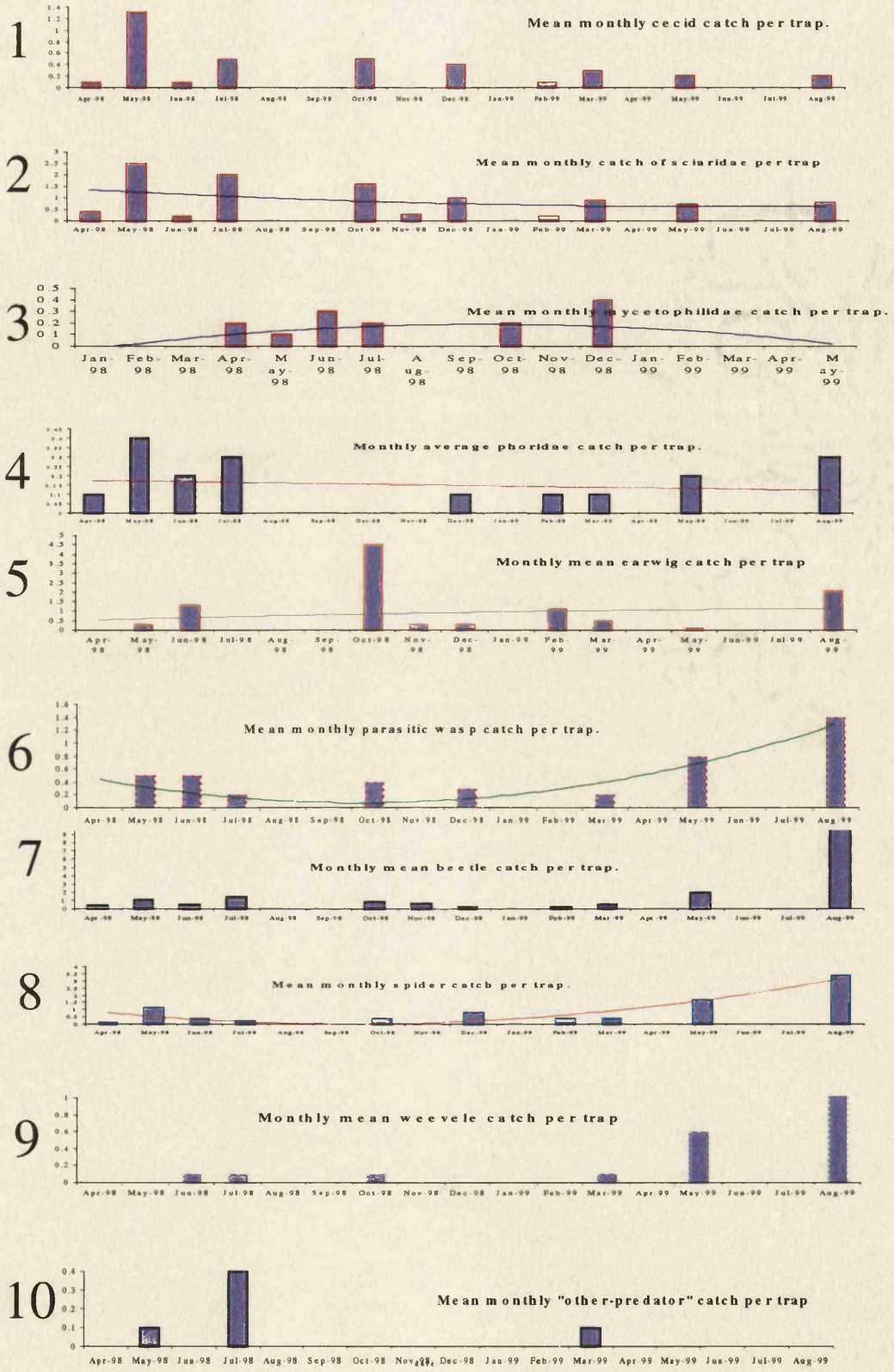


Figure 3.20: Field trap data displayed as total catch for 10 different groupings of insect and invertebrate taxa from Cecids (1), Sciarids (2), Mycetophilids (3), Dhorids (4), earwigs (5), parasitic wasps (6), beetles (7), spiders (8), Lepidoptera (9), weevles (10) and other-predators (11). All organisms emerged from approximately 0.025m^3 of decaying *Corylus avellana* wood.

Table 3.7 Insect trap correlation (n=140) (see appendix 5 for raw catch data / trap / month)

CORRELATION Strength

	cecids	sciaridae	mycetophilidae	phoridae	beetles	spiders	earwigs	parasitic wasp	lepidoptera	weevele	aphid	other predators
cecids	1	* **0.66**	0.073	**0.228**	0.028	0.044	**0.351**	**0.128**	0.121	-0.049	**0.272**	**0.145**
sciaridae		1	**0.392**	**0.176**	**0.347**	0.078	**0.336**	**0.11**	0.174	0.013	**0.221**	**0.15**
mycetophilidae			1	**0.165**	-0.014	-0.013	-0.009	0.032	-0.058	-0.023	**0.171**	-0.055
phoridae				1	0.277	0.002	0.063	**0.242**	0.239	**0.191**	**0.571**	**0.152**
beetles					1	* **0.709**	**0.255**	* **0.59**	0.205	* **0.503**	**0.108**	**0.354**
spiders						1	**0.28**	**0.441**	0.215	**0.244**	**0.12**	**0.284**
earwigs							1	**0.276**	-0.012	0.083	-0.04	**0.495**
parasitic wasp								1	0.259	* **0.644**	**0.104**	* **0.562**
lepidoptera									1	**0.486**	-0.026	**0.269**
weevele										1	0.029	**0.443**
aphid											1	-0.003
other predators												1

Key:
bold = slope > 0.1
* and **bold** = slope >0.5

CORRELATION P.nos

	cecids	sciaridae	mycetophilidae	phoridae	beetles	spiders	earwigs	parasitic wasp	lepidoptera	weevele	aphid	other predators
cecids	Zero	* **0.0001**	0.448	* **0.017**	0.768	0.65	* **0.0001**	0.182	0.208	0.609	* **0.004**	0.13
sciaridae		Zero	* **0.0001**	0.066	0.347	0.417	* **0.0001**	0.253	0.068	0.982	**0.02**	0.118
mycetophilidae			Zero	0.085	0.886	0.895	0.924	0.742	0.55	0.811	0.074	0.56
phoridae				Zero	**0.003**	**0.002**	0.512	* **0.011**	**0.012**	* **0.012**	**0.0001**	0.112
beetles					Zero	* **0.0001**	* **0.007**	* **0.0001**	**0.032**	* **0.0001**	0.263	* **0.0001**
spiders						Zero	* **0.003**	* **0.0001**	**0.024**	* **0.01**	0.903	* **0.003**
earwigs							Zero	* **0.004**	0.9	0.387	0.68	* **0.0001**
parasitic wasp								Zero	**0.006**	* **0.0001**	0.281	* **0.0001**
lepidoptera									Zero	* **0.0001**	0.784	* **0.004**
weevele										Zero	0.761	* **0.0001**
aphid											Zero	0.971
other predators												Zero

Key:
bold = P <0.05
*and **bold** = P < 0.05 + in other table

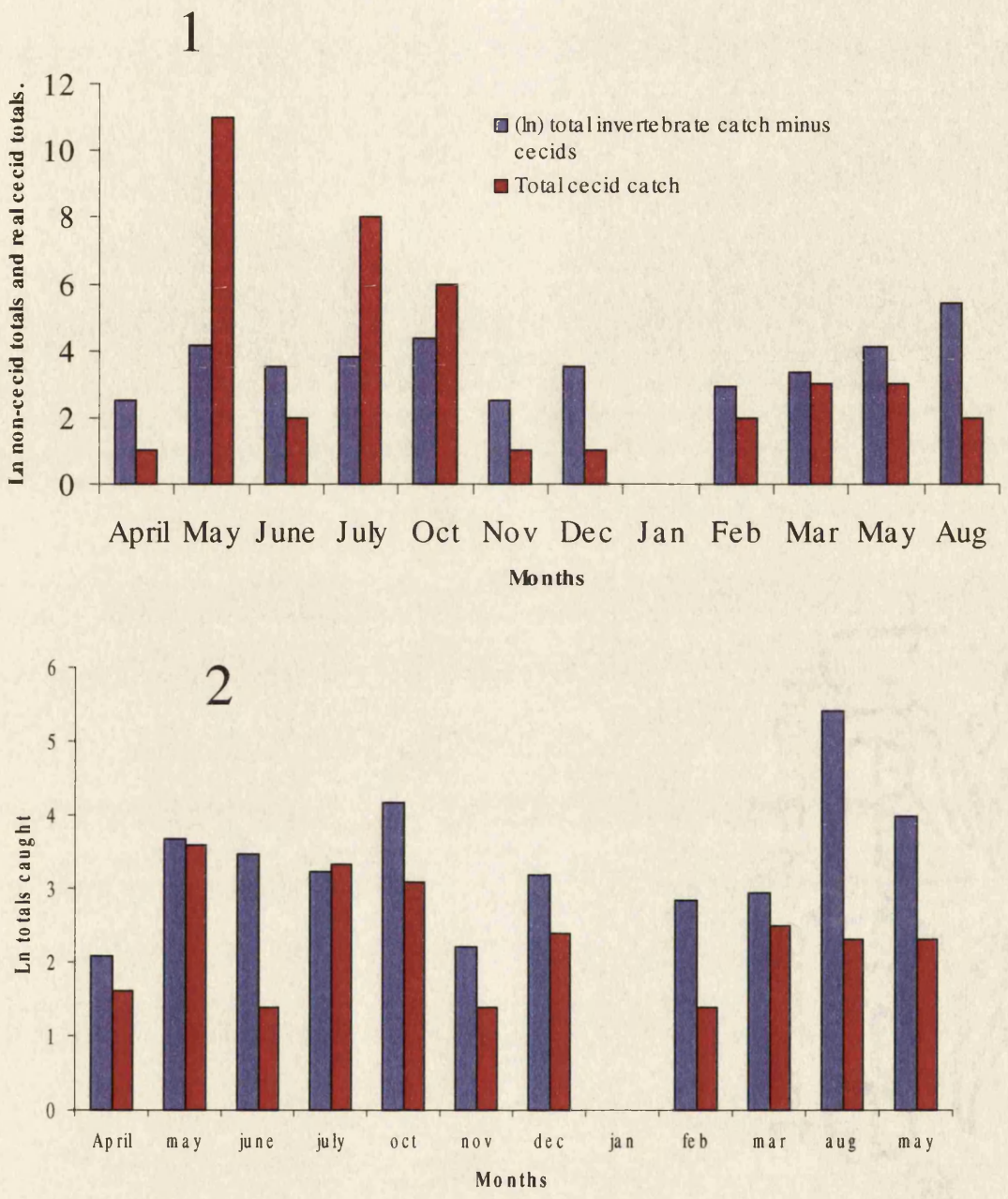
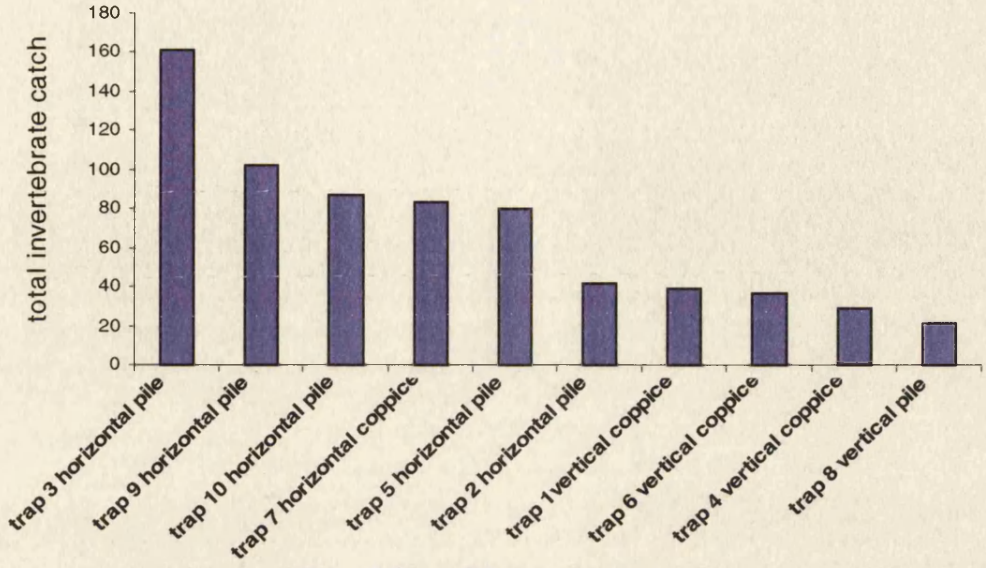
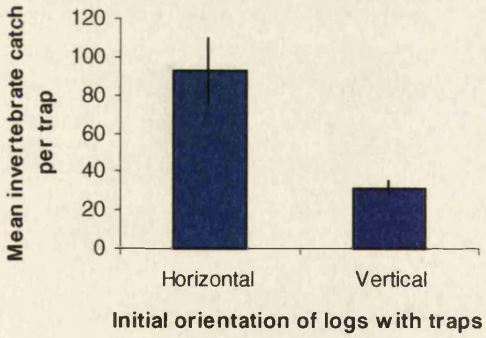


Figure 3.21: (1) shows totals for cecids and non-cecids as a comparison of emergence. (2) shows cecids and sciarids as one group of diptera, in comparison with the rest of the invertebrates trapped.

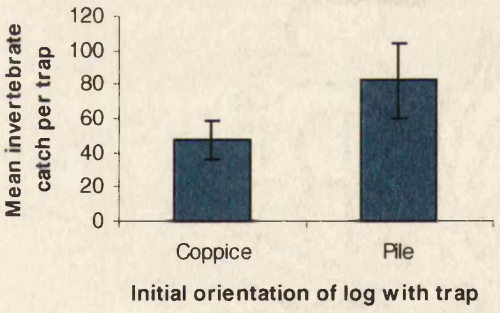
1 **Total invertebrate catches ranked according to initial orientation of logs vertically or horizontally and whether attatched to coppice or in log pile.**



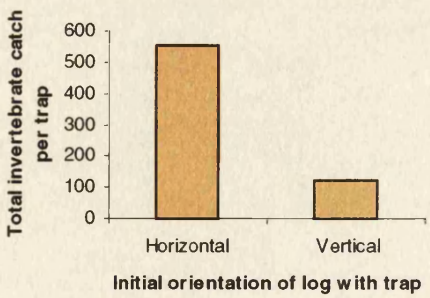
2 **Comparison of mean invertebrate catch between vertical and horizontal logs. (n=5, P<0.001)**



4 **Comparison of mean invertebrate catch between logs attatched to coppices and traps in log piles. (n=5, P<0.05)**



3 **Comparison of total invertebrate catch between logs in different initial orientations**



5 **Comparison of total invertebrate catch between logs attatched to coppice or in log piles**

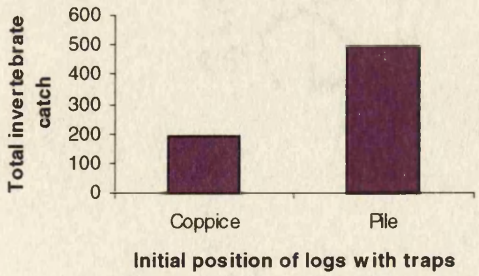
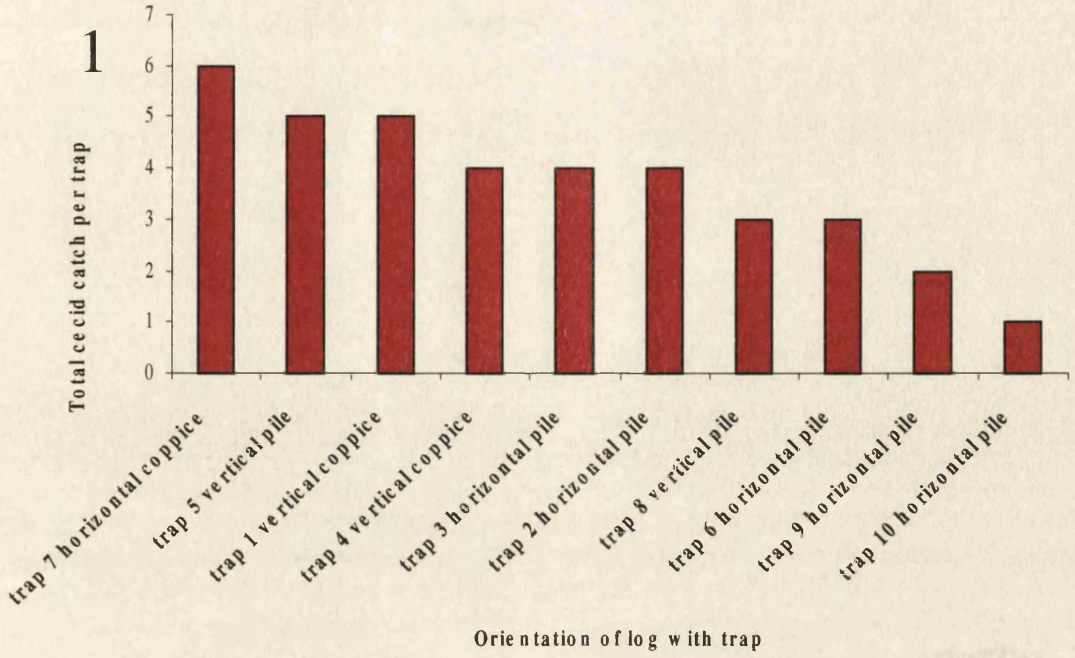
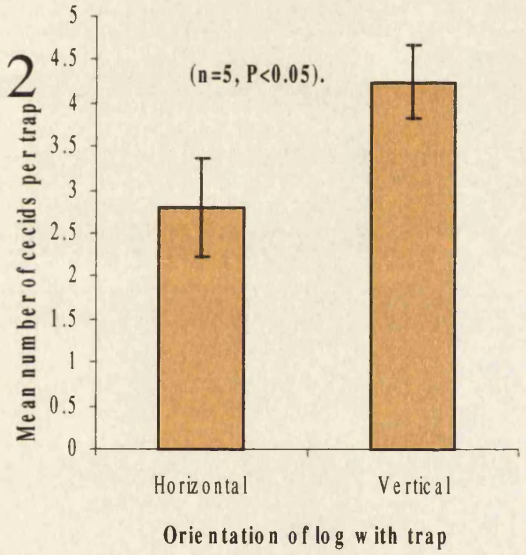
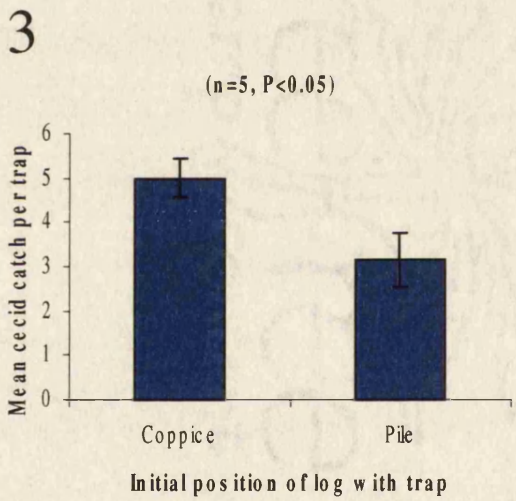
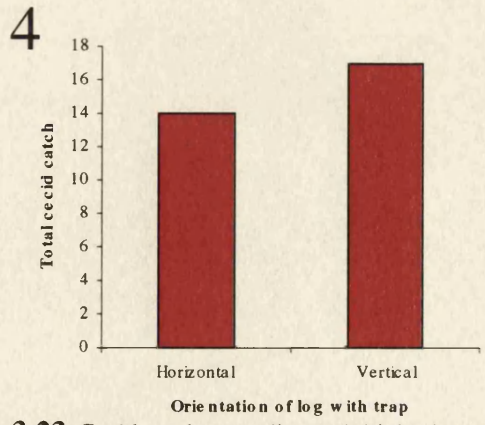


Figure 3.22 Total invertebrate catch according to initial orientation and position of logs in woodland (1). (2) and (3) compare catches between orientations, (4) and (5) compare catches between positions. Standard error bars are shown in mean data pots (2) and (4).









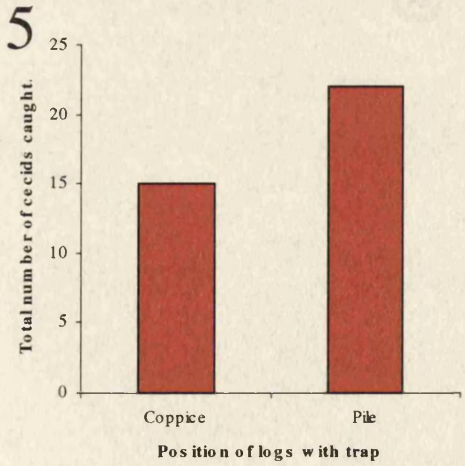


Figure 3.23: Cecid catch according to initial orientations and positions of logs in woodland. (1) shows each trap's cecid catch in terms of orientation (vertical or not) and position (in pile or coppice). (2) shows comparison of means for orientation with standard error bars (n=5, P<0.05), (3) shows comparison of means for position with standard error indicated (n=5, P<0.05), (4) and (5) show total catch data for orientation and position.

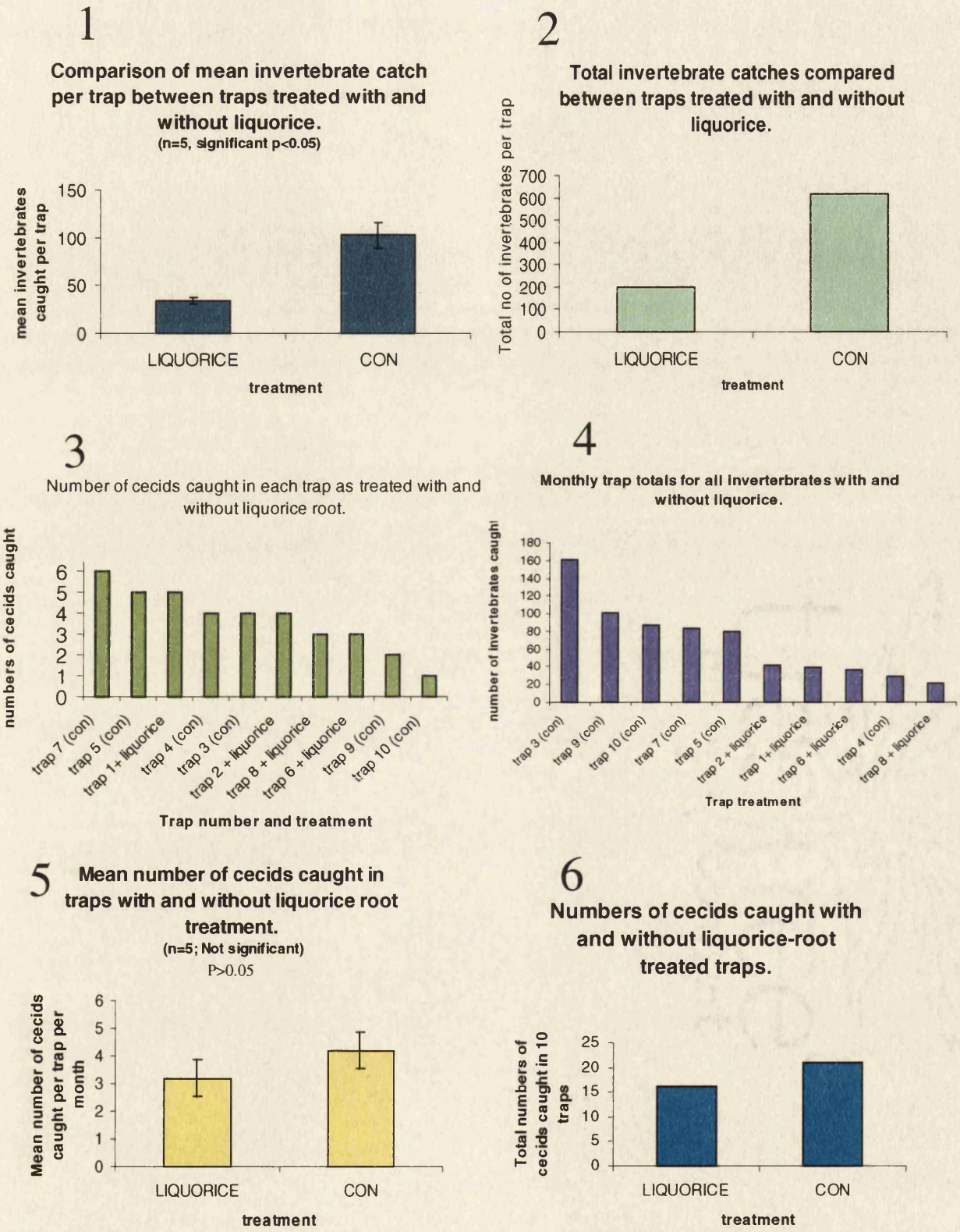


Figure 3.24: Comparisons of invertebrate catch results for traps treated with and without liquorice root. (1) shows an all-organism comparison of means with standard error bars marked, (2) shows total organism catch comparisons, (3) shows cecid-data for each trap labelled as liquorice or not, (4) shows all organism-data labels as liquorice or not. (5) shows cecid comparison of means with standard error bars marked. (6) shows total cecid catches between liquorice and control traps.

samples, significantly more invertebrates ($P<0.001$) were caught from horizontal logs than vertical ones. Furthermore, significantly greater invertebrate catches were obtained from logs in piles rather than in a natural coppice ($P<0.05$). Figure 3.23 shows cecid catches according to log orientation. Here significantly more cecids (through the Mann-Whitney test) were trapped in vertically orientated logs ($P<0.05$), and in natural coppice stands ($P<0.05$) than in log piles.

Figure 3.24 shows graphs 3 and 4 which compare cecids and all invertebrates for the test with liquorice in four of the traps. Graphs 1 and 2 show the significance (confirmed with Mann-Whitney test) of fewer invertebrates in total trapped in the "liquorice traps" ($P<0.05$). However, data on cecids only, presented in graphs 5 and 6, shows no significant difference ($P>0.05$) between liquorice and non-liquorice containing traps.

3.4.7 Microscopy and larval identification - results

Polarised light micrographs revealing creeping welts and some trachea of the field cecid most commonly encountered in the study site are shown in figures 3.3. Scanning electron micrographs of the field cecid are presented in figures 3.25, 3.26, 3.27, 3.28, 3.29 and 3.30 which yielded evidence, on the basis of Wyatt's key, of the identity of field cecid colonies as being *Brittenia fraxinicola* (Wyatt 1967). As far as I can establish, these are the first scanning electron micrographs of this family Heteropezini, genus *Brittenia sp.*, species *fraxinicola*. One of the indicative features which enabled *B. fraxinicola* to be identified were 13 spinule rows of ventrally positioned creeping welts, shown in close up in the scanning electron micrograph in figure 3.28. In figure 3.26 dorsal and ventral creeping welts can be seen on anterior larval segments (Wyatt 1967). Also another indicative feature for *B. fraxinicola* is visible in figure 3.26: that of localised anterior spinules just anterior to the spinule rows that collectively make up the creeping welts (Wyatt 1967, and introductory figure 3.4). Further evidence confirming the identification as *B. fraxinicola* are the posterior sensoria just behind creeping welts which are also visible in figure 3.26. However, identification could not be confirmed until the head and anus had been examined for external features as seen in figure 3.27. Here, the shape of the palps, mandibles and 6 pronged, four-lobed anus confirms identification as *Brittenia fraxinicola* [Edwards] (figure 3.4). Figure 3.28 shows scanning electron micrographs of *B. fraxinicola* showing the size difference between the 2 instars. It has been suggested from work on other species that a cecid hemi-pupa can lie with dormant first-instars inside for long durations of adverse conditions (Wyatt 1929) up to 12 years long (Mamaev & Krivosheina 1993). Figure 3.29 shows a fungal condition of *B. fraxinicola* observed occasionally in the field, initially as swellings in the cuticle, later as sporophores of a fungus, usually sprouting from hemi-pupae *in situ* under bark layers. Each swelling on the edge of hyphae is a type of reproductive conidiophore, like a yeast, which could possibly be carried off in water or by passing invertebrates. Figure 3.30 shows a scanning electron micrograph of a much larger non-paedogenic, probably predatory orange cecid larval species occasionally found in the same niche as *Brittenia fraxinicola.* This species could not be cultured successfully on fungi in the laboratory. It is possible that these developed into the cecid adults, which were caught in the traps. A photograph of this orange larval species, lying on its dorsal surface having been freshly exposed by peeling back a piece of bark, can be seen at the bottom of figure 3.31. This also shows *B fraxinicola in situ* inside pseudosclerotial plate cavities between layers of bark and medullary ray protrusions.

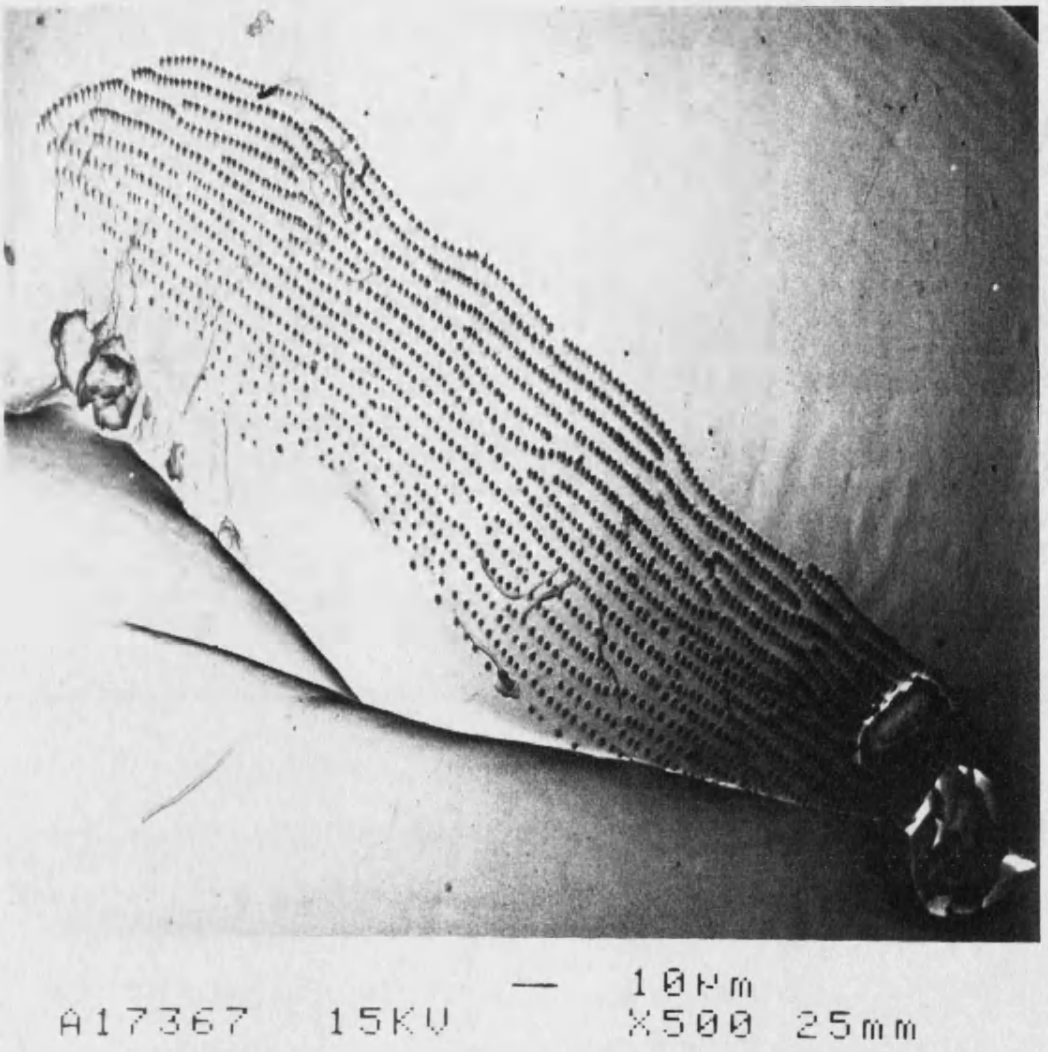


Figure 3.25 Creeping welts of the field cecid *Brittenia fraxinicola* consisting of 13 spinule rows on the anterior ventral surface of each larval segment.

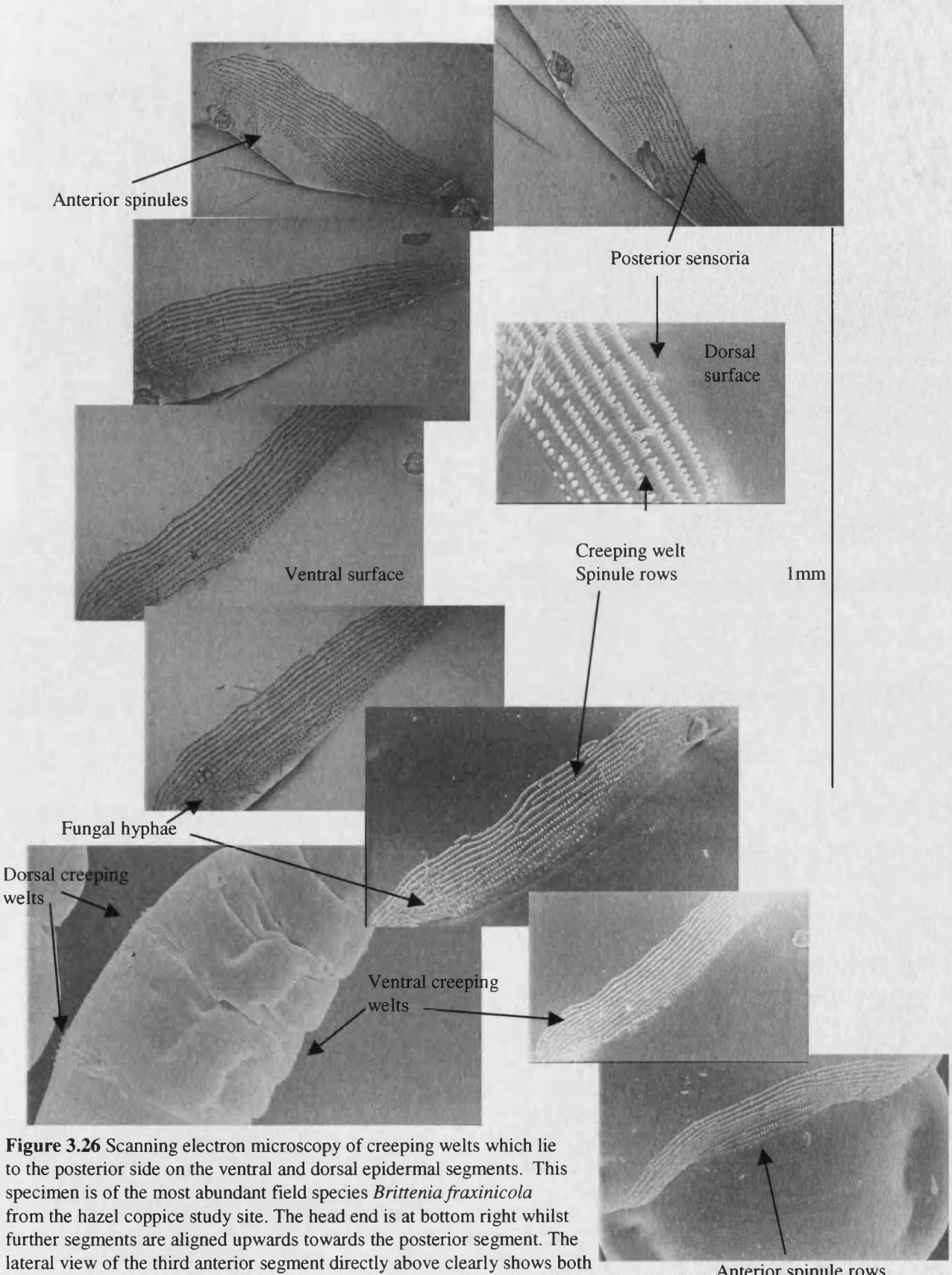


Figure 3.26 Scanning electron microscopy of creeping welts which lie to the posterior side on the ventral and dorsal epidermal segments. This specimen is of the most abundant field species *Brittenia fraxinicola* from the hazel coppice study site. The head end is at bottom right whilst further segments are aligned upwards towards the posterior segment. The lateral view of the third anterior segment directly above clearly shows both ventral and dorsal creeping welts, spinules and sensoria (arrowed). Rows of sensoria can be seen just posterior to creeping welts, most clearly in the close up at middle right .

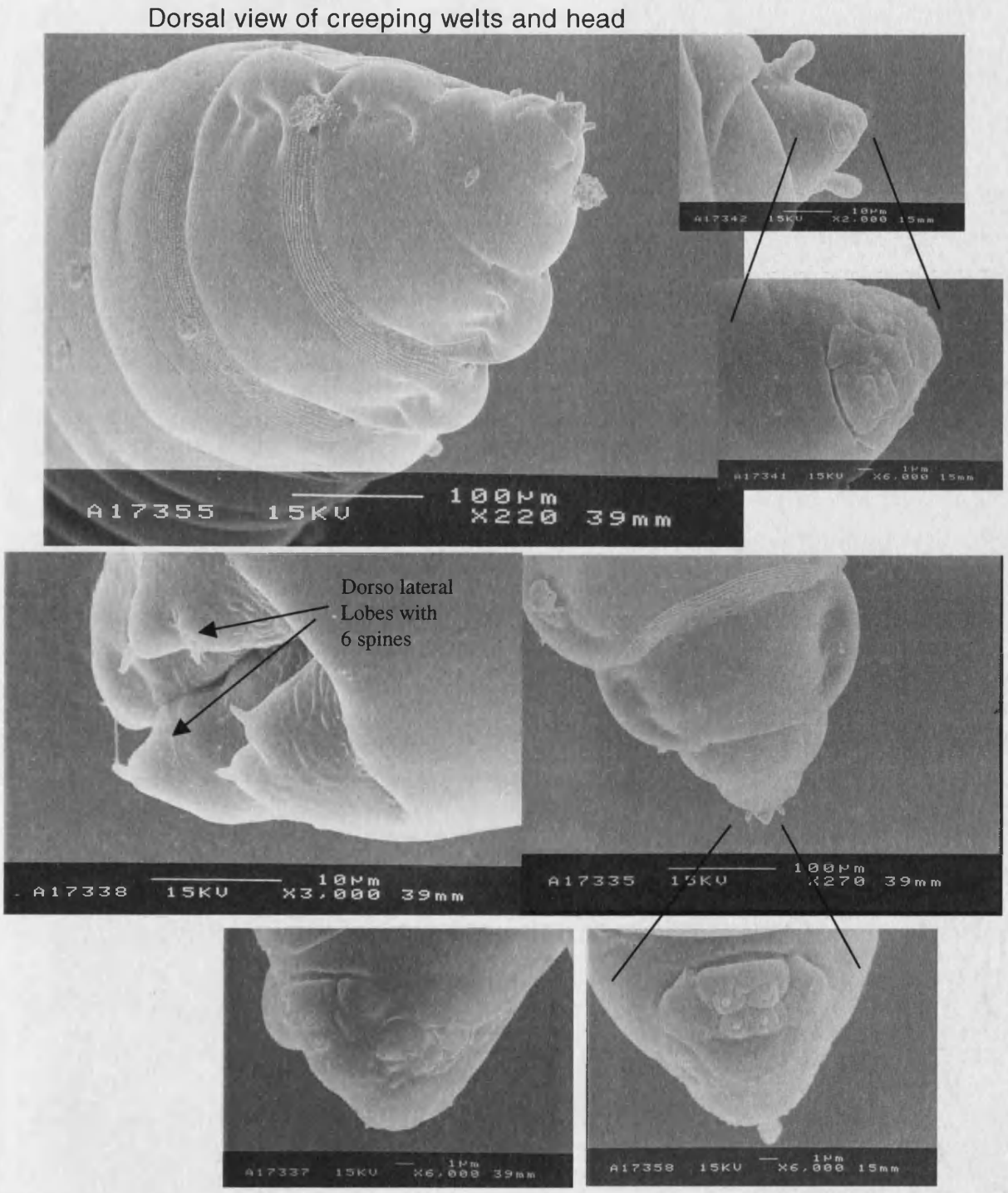


Figure 3.27: Scanning electron microscopy of the abundant field species *Brittenia fraxinicola* showing details of head, mouthparts and anus (at middle left). Also a good view of dorsal creeping welts on 2nd + 3rd thoracic Segment. 6 th anal spine is hidden by a fold in the posterior dorso-lateral lobe.

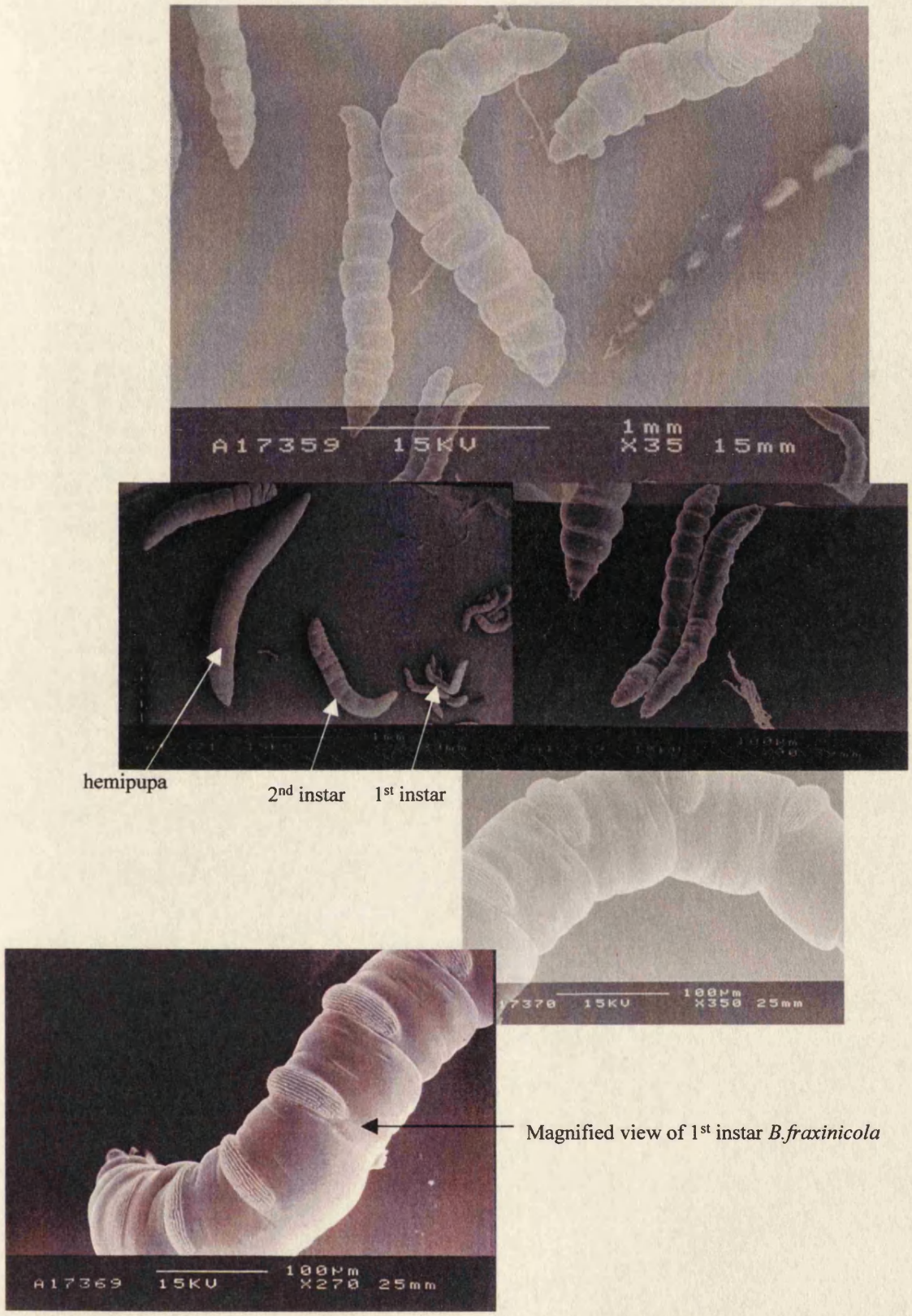


Figure 3. 28 Scanning electron microscopy of abundant *Brittenia fraxinicola* larvae showing different views at various magnifications of 1st and 2nd instars and extensive swelling of creeping welts in 1st instars enabling them to stick to almost any surface – including themselves, sometimes forming large clumps.

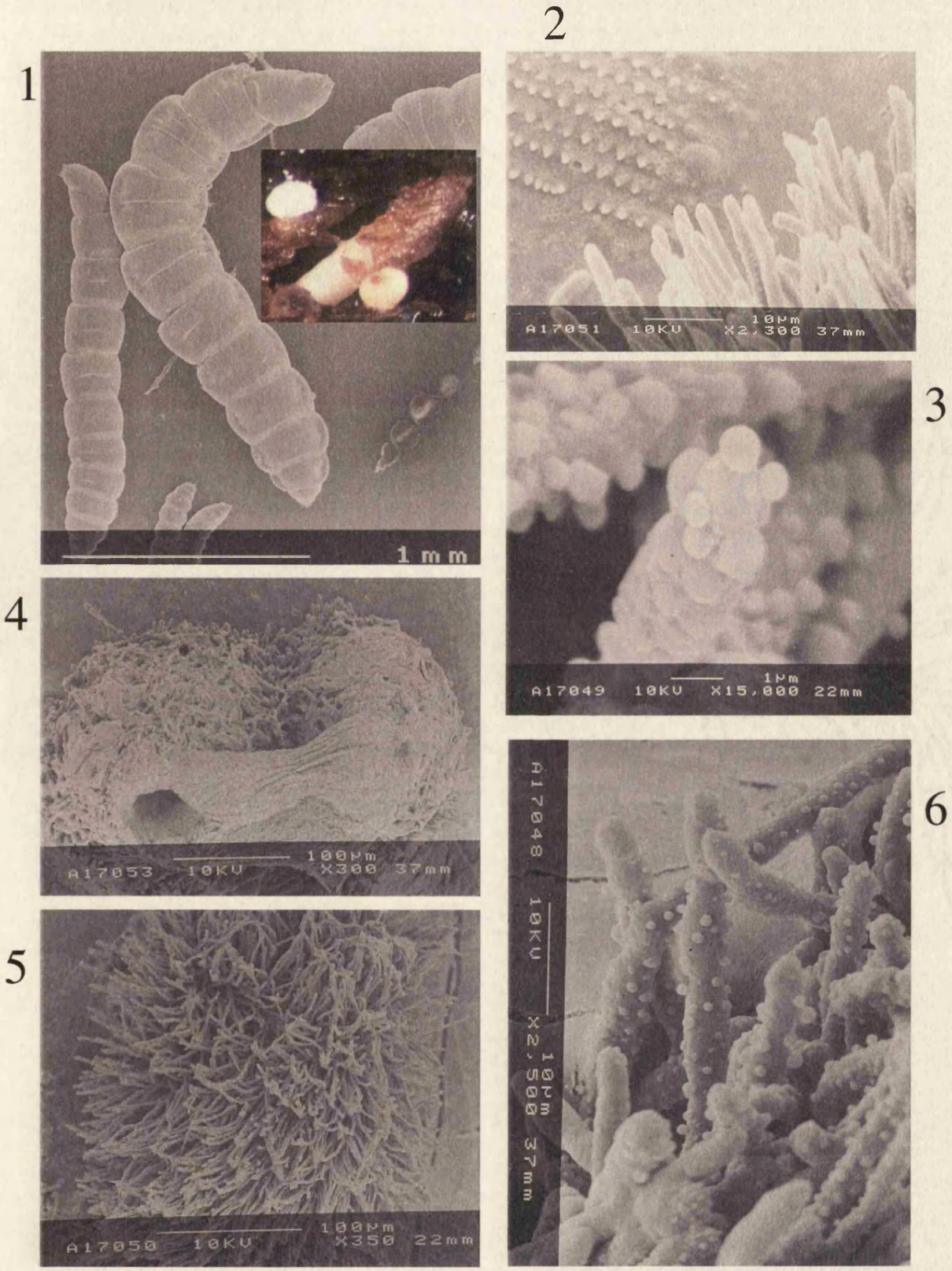


Figure 3.29 (1) Particular developmental stages of field cecids *Brittennia fraxinicola* were found in mid August 1997 and in 1998 to have an occasional incidence of fungal growths erupting from pustular swellings (1-inset) in the hemipupal epidermis (2 and 5). These scanning electron micrographs show some mycelial characteristics of these fungi. Budding of small asexual spores from lateral hyphae (3) and (6) would make this fungus very contagious if it were entomopathogenic.

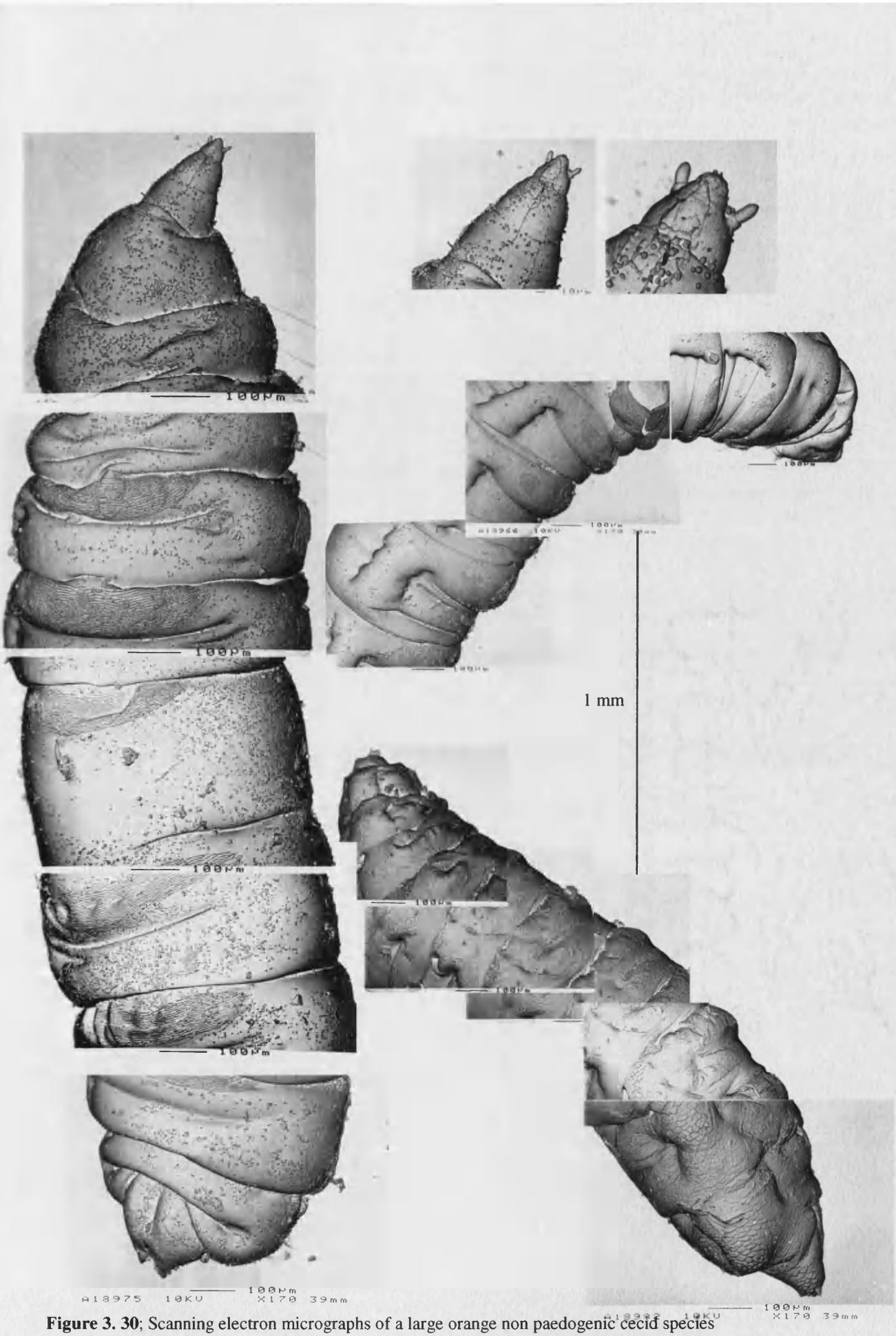


Figure 3. 30; Scanning electron micrographs of a large orange non paedogenic cecid species found in the field under bark of logs undergoing latter stages of decomposition. Notice the spores and hyphae covering it's cuticle surfaces.

3.4.8 Cavity measurements - results

Observations (figure 3.31) of smaller *B. fraxinicola* larvae being able to escape predation by fitting into tighter clefts in the bark matrix into which larger cecids could not fit, led me to consider the implications of cavity dimensions and the size of organisms found therein. Figure 3.31 shows cecids found inhabiting the fractal cavity architecture of the sub-cortical bark complex of decaying *Corylus avellana*. Fractal organisation of bark and grass-dwelling organisms (Wilson 1992 a, Clay 1988, Lane 1998) frames the theme of the next set of results from measuring emergent architectural details of the spaces in which organisms were found. As shown in figure 3.32, layers of dead bark form a complex of inter-connected spaces in which invertebrates, especially insect larvae, inhabit. The cavities in which larvae are found can be measured in terms of width and height. Figure 3.32 shows significant correlation ($P < 0.05$) between length of lifted bark and length of PSP at the bark-sapwood interface, bark thickness and cecid sizes (in width and length) ($P<0.05$), bark thickness and cavity width ($P<0.01$), cavity height ($P<0.01$) and a function of height x width (equivalent to "entrance area", the area encountered by an insect trying to enter into such a cavity) ($P<0.05$). Further significant correlations between cavity measurements and cecid dimensions are shown in figure 3.33. In a more general context, figure 3.34 places cecid width correlation data alongside that for other invertebrates found under bark (bottom graph). Here significant correlations ($P<0.05$) were found for cecids and non-cecids whether correlating to cavity width ($P<0.001$) or bark thickness ($P<0.01$) (figure 3.35). Non-cecid lengths and widths both increased significantly ($P<0.05$) with cavity width, as shown in graphs in figure 3.36. Figure 3.37 shows a significant correlation ($P< 0.005$) between the number of fungal domains in a log and the amount of PSP at the bark-sapwood interface (1). This figure also shows a significant correlation ($P<0.005$) between the amount of lifted bark with cavity complexes underneath, and the number of fungal decay columns, or genets (2). These results suggest that the process of sub-cortical cavitation is influenced by the amount of PSP zone. Ecological succession in decay of hazel also is shown to correlate significantly with increasing cavity-size ($P<0.005$). Figure 3.38 (1) shows ranked order of fungal decay in terms of ruderal to K-selected species in order of colonisation from *Hypoxylon fuscum*, to *Hymenochaete corrugata*, then *Daldinia concentrica* and finally the K-selected basidiomycete chord-former *Armillaria mellea*. The cavity widths measured under bark colonised by these mycelia are shown on the y-axis. Graph 2 shows a significant correlation ($P<0.005$) between size of woody debris (diameter) and cavity-size for three tree species. Graph 3 shows a similar trend between cavity-size with tree species ranked in terms of ruderal towards K-selected from pioneering *Corylus avellana* to *Fraxinus sp.* and climactic *Quercus sp.* ($P<0.005$).

3.4.9 Cecid "jumping" behaviour results

Figure 3.39 shows the first photographic evidence of airborne cecids in the act of "jumping" from a dense cluster of mainly first-instar *B. fraxinicola* larvae emerging from under bark after summer rain in July 1999. Many of these larvae are photographed actively flinging themselves from initially coiled positions from the bark layer where a cut was made. Sections of bark containing cecids had been placed in the laboratory "jumping-arena" shown in figure 3.39. Data from this novel technique for measuring larval dispersal distance and height "jumped" are shown in figure 3.40. Distance trials are shown in graphs 1,2,3,4,5 & 6. Graph 1 is for cecids "jumping" from a small piece of bark suspended vertically 10 cm above the arena. Some larvae from this height "jumped" a distance of 0.5m. Graphs 2,3,4 & 5 are for cecids that "jumped"

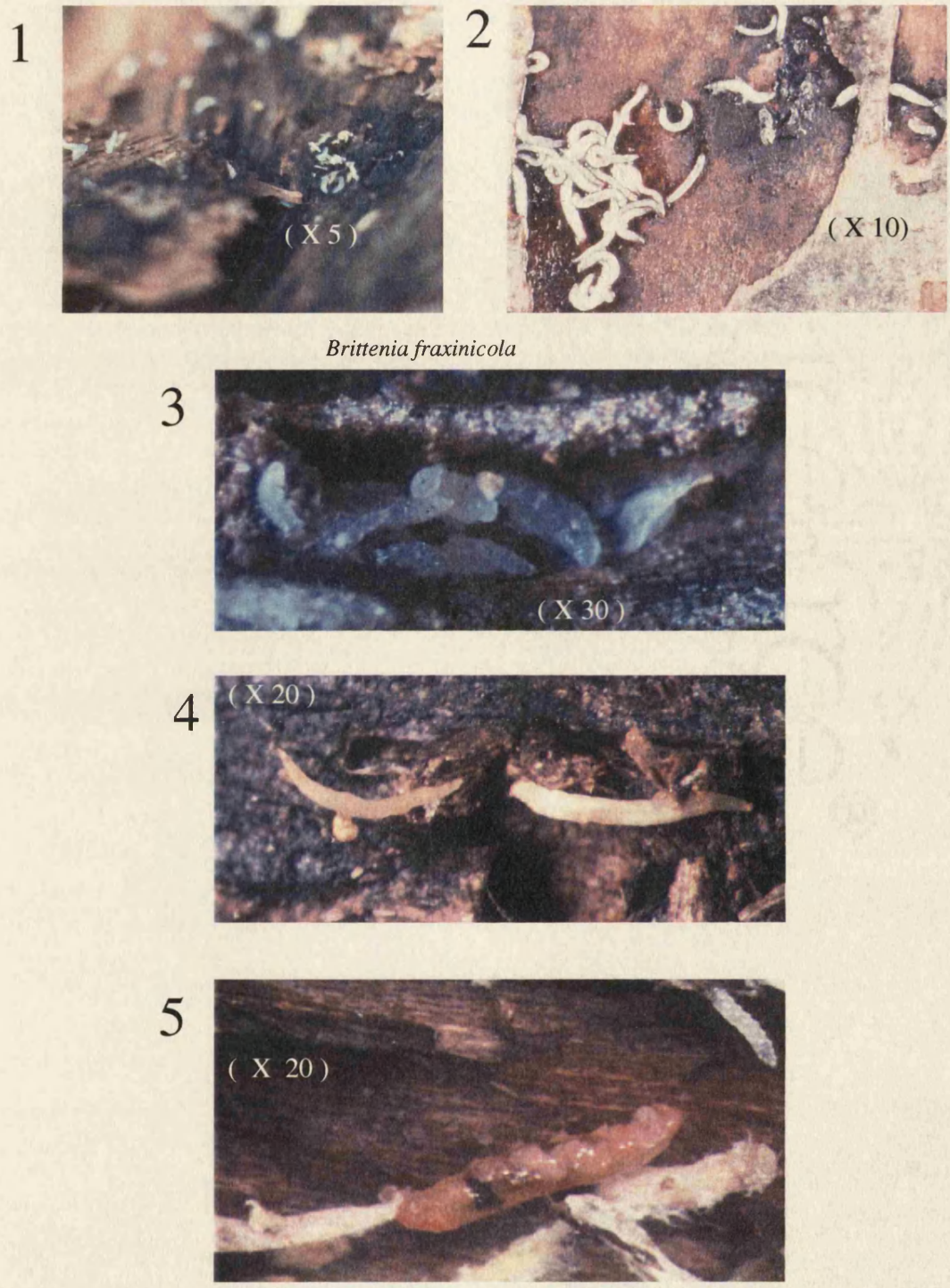
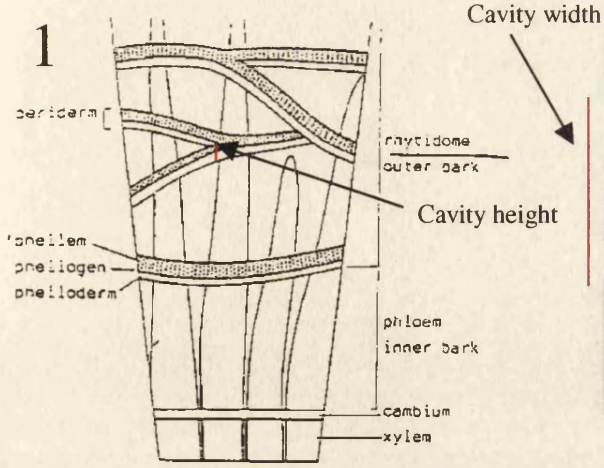
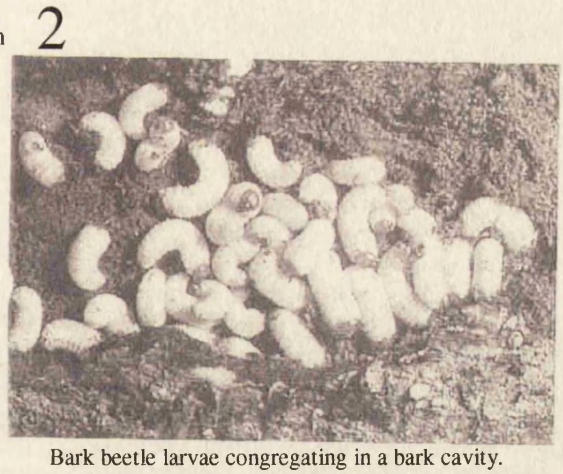


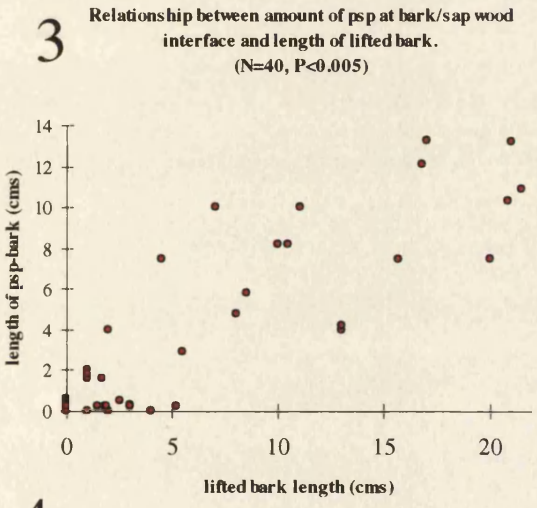
Figure 3.31 Field cecids photographed using a macro-lense in situ, having been observed under freshly peeled bark; (1), (2), and (3) show common study site cecid; *Brittenia fraxinicola*. (4) shows a less common field cecid of genus *Lestremiinae* sp. (5) shows a non paedogenic orange and larger field cecid of unknown genus, far lesser density, which is perhaps predatory (figure 3.31).

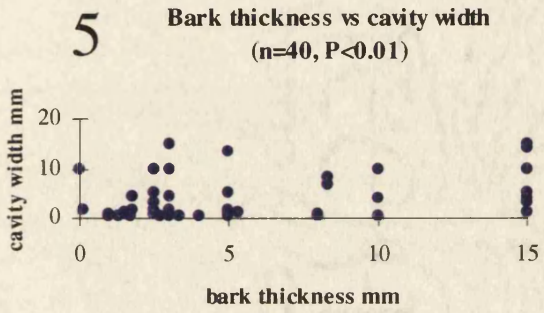


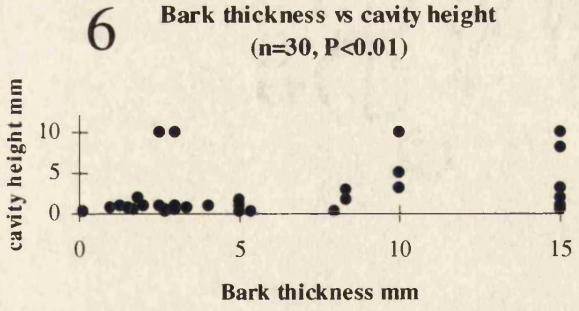
Schematic of general bark structure (from Junikka 1994)

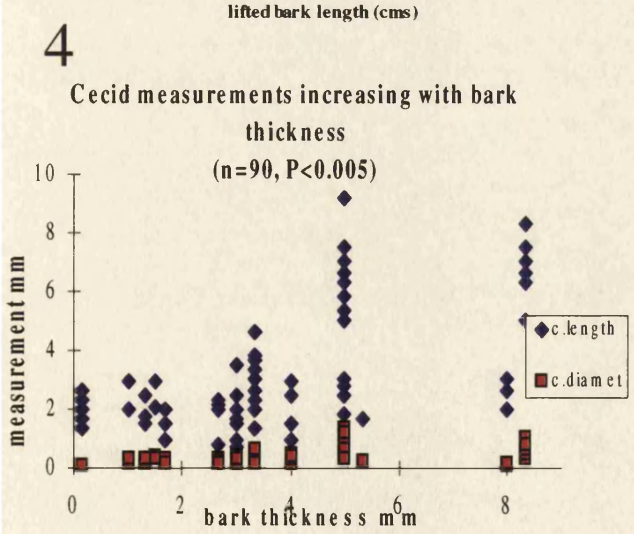


Bark beetle larvae congregating in a bark cavity.
(from Speight, Hunter and Watt 1999)









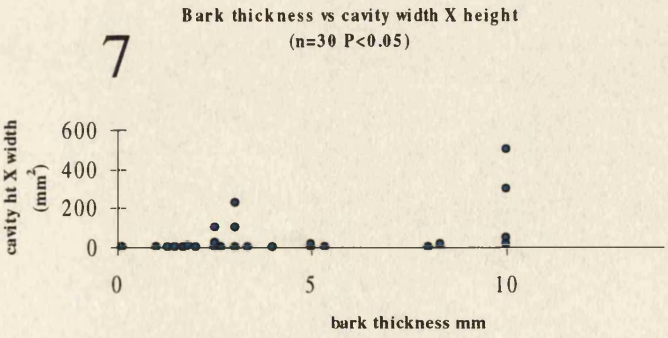
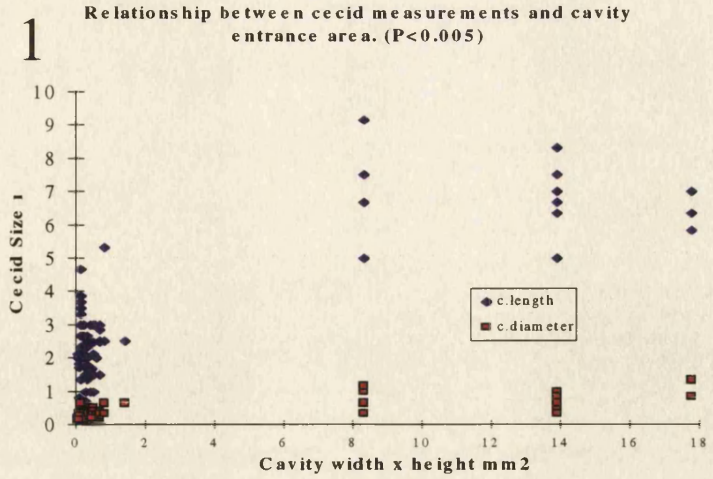
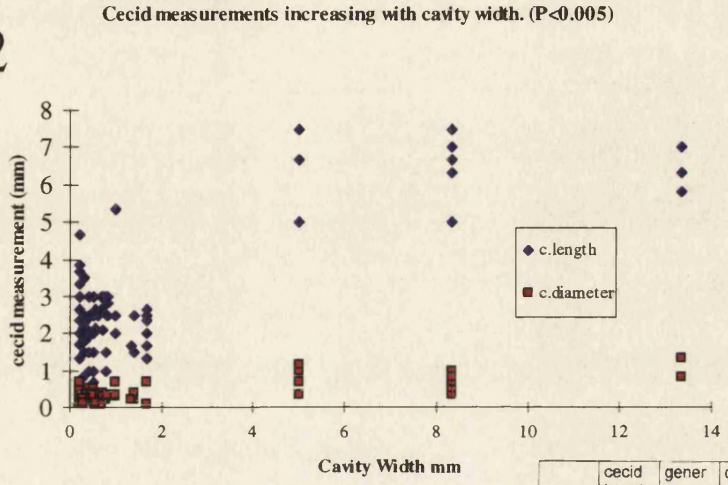


Figure 3.32 Schematics at top (1) illustrate bark matrix and potential for insect larvae to cluster in cavities thereof (2). Graphs below demonstrate relationships between lifted bark and length of PSP at bark (3), bark thickness and cicid dimensions (4), bark thickness and cavity widths (5), bark thickness and cavity heights (6) and bark thickness with a function of cavity width x height (area of cavity entrance in cm^2) (7).

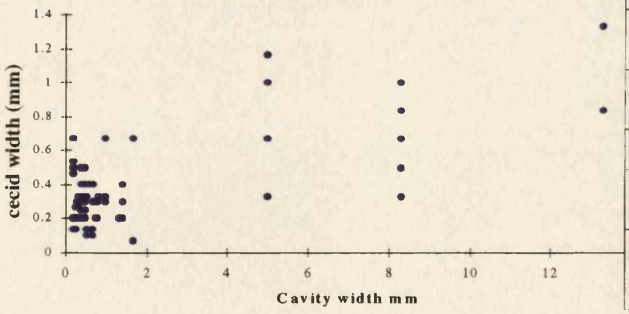
1



2



3



4

	cecid larval width	genera insect width	cavity width	cavity height	cecid larval length	cavity length	genera insect length
cavity width	Y	Y	-	Y	No	No	No
cavity height	Y	Y	Y	-	No	No	No
cavity width x height	Y	Y	Y	Y	No	No	No
thickness of bark	Y	Y	Y	Y	No	No	No
cavity length	No	No	No	No	No	No	No
cavity width x length	No	No	No	No	No	No	No
cavity length x height	No	No	No	No	No	No	No

Figure 3.33 Significant correlation between measurements of cecid larvae and cavity entrance areas (1), and cavity widths (2) and (3). Table (4) shows other cavity correlations. Yes = P<0.05, No = P>0.05.

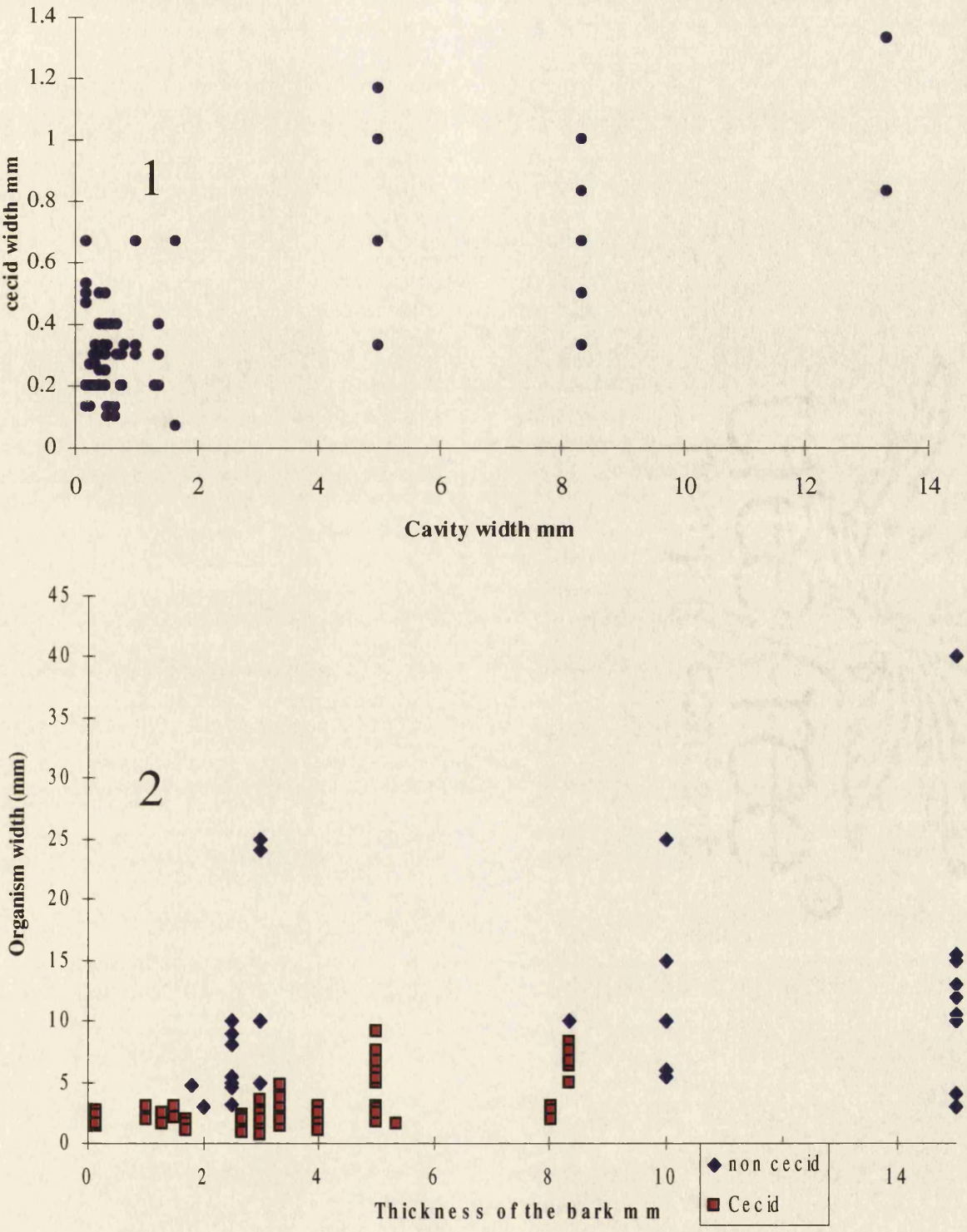
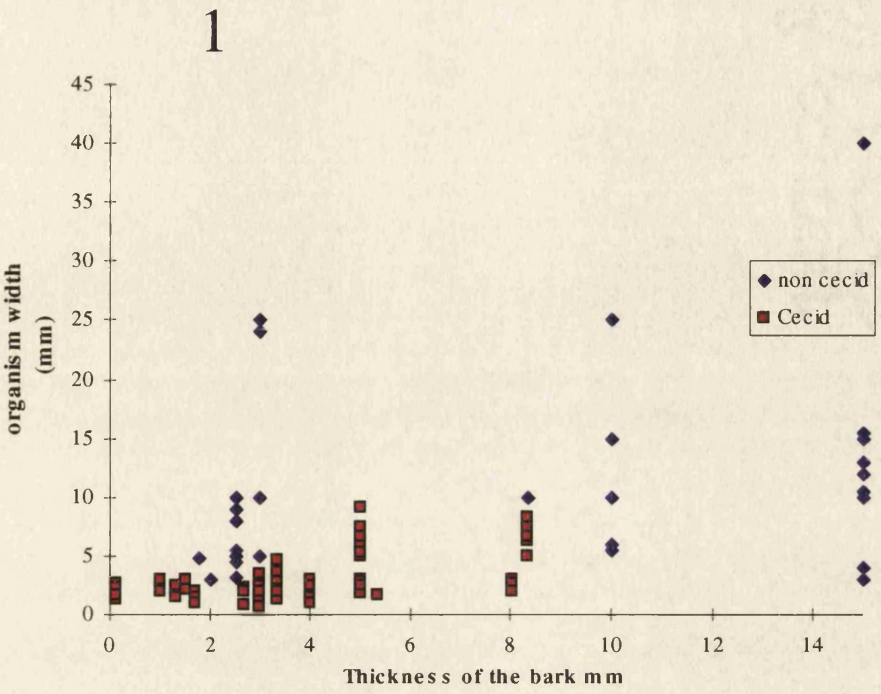


Figure 3.34; (1) shows the relationship between width of sub-cortical cavities and widths of cecids found within them (P<0.001). (2) shows relationship between thickness of bark and width of cecid and other organisms found in sub-cortical zones (P<0.01).



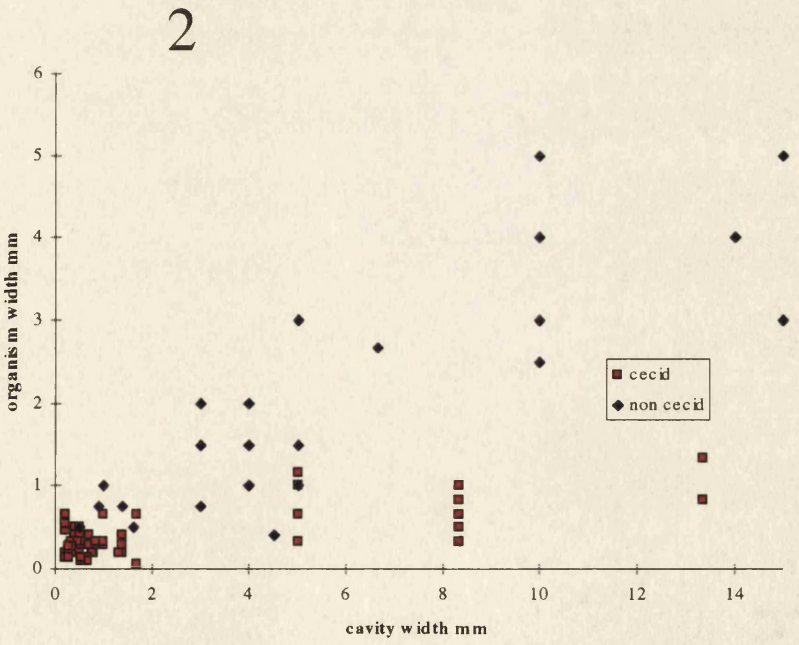


Figure 3.35 (1) significant correlation between separated data sets of cecid and non-cecids with thickness of bark (P<0.01). (2) shows a stronger relationship with cecid and non-cecid measurements plotted against cavity width (P<0.001).

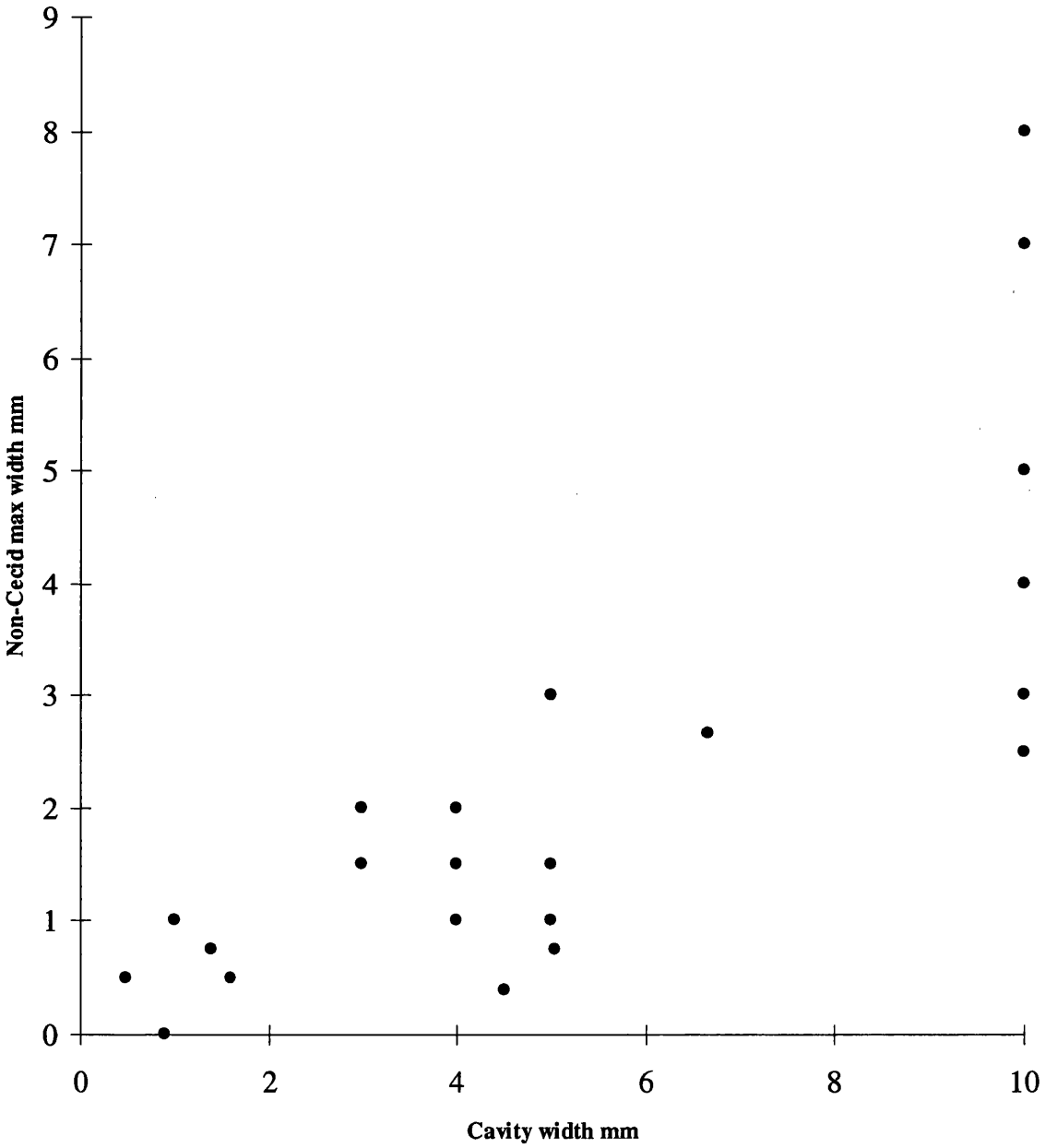


Figure 3.36 Shows a significant (P<0.005) correlation between cavity width and non-cecid organism dimensions.

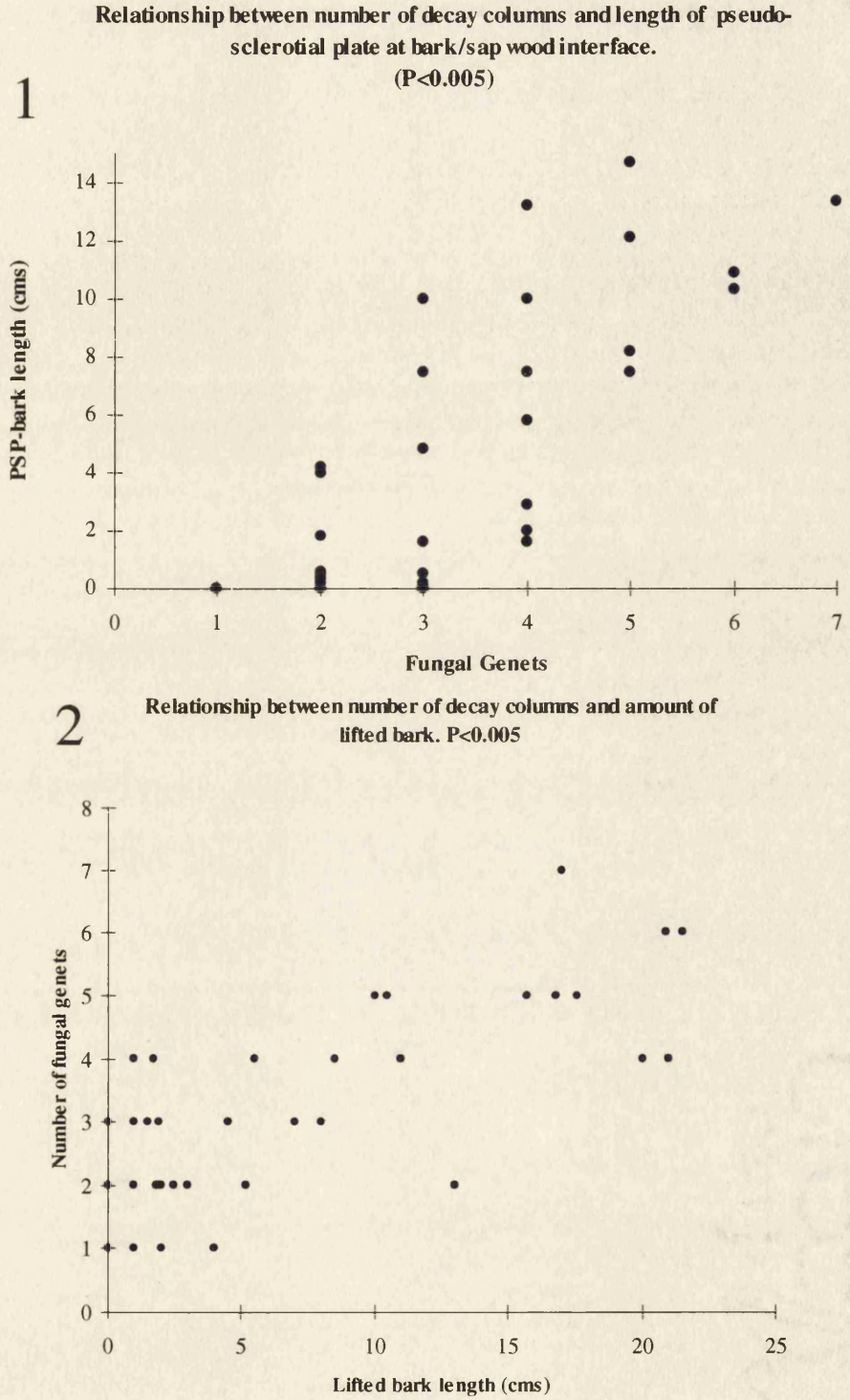


Figure 3.37 Significant correlation (P<0.005) between, (1) number of fungal genets in a log and the amount of PSP at bark-sap wood interface. (2) shows a strong correlation between number of fungal genets and amount of bark-lift (P<0.005).

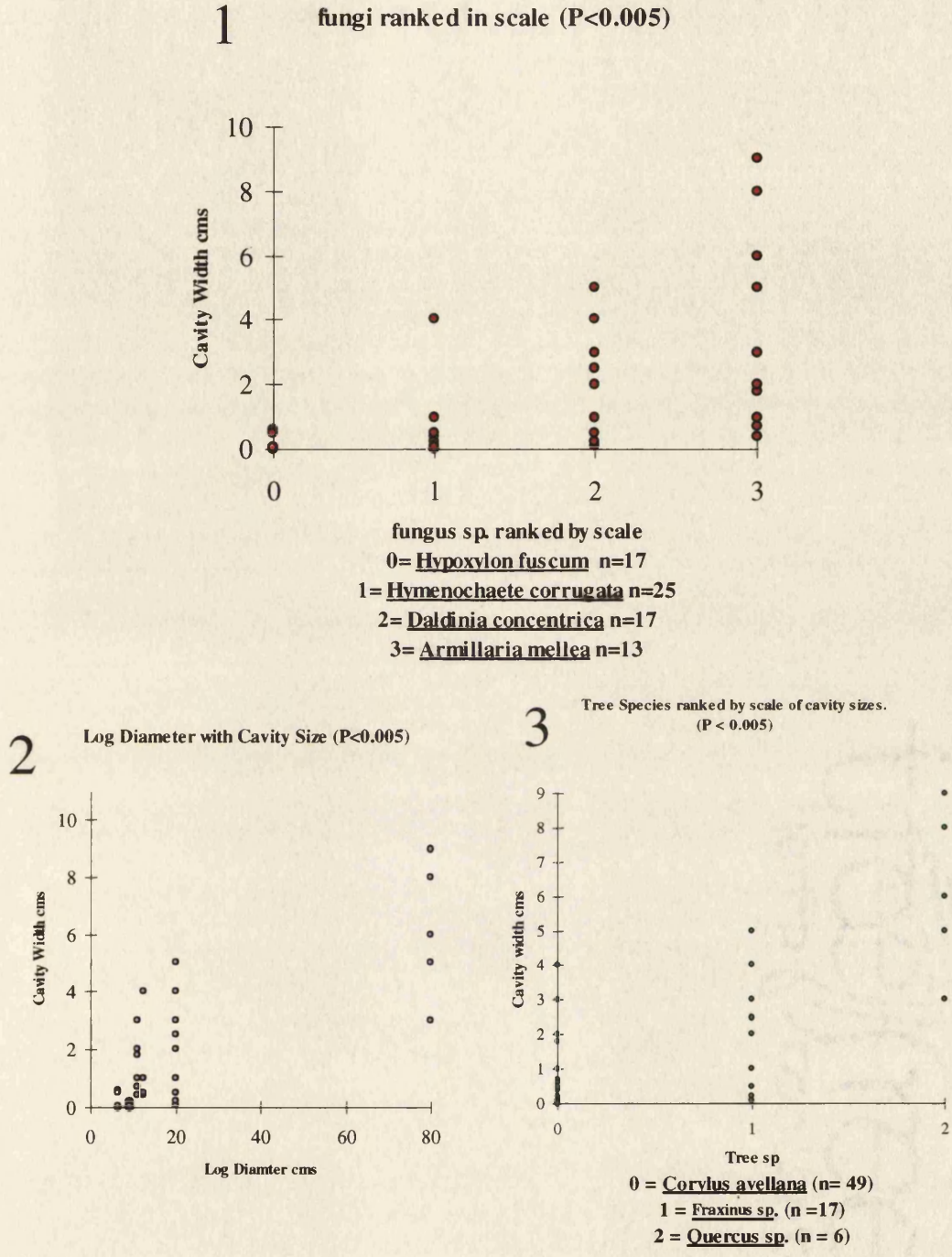


Figure 3.38 (1) shows a significant correlation through an r-K ranked group of 4 fungal species found in rotting log piles. The fungal species have been ranked along the x-axis in order of increasing K-selectedness (and scale). (3) shows a similar correlation by r-K ranking of tree species. (2) shows the relationship between log diameter and cavity widths found within sub-cortical zone to be significant (P<0.005).

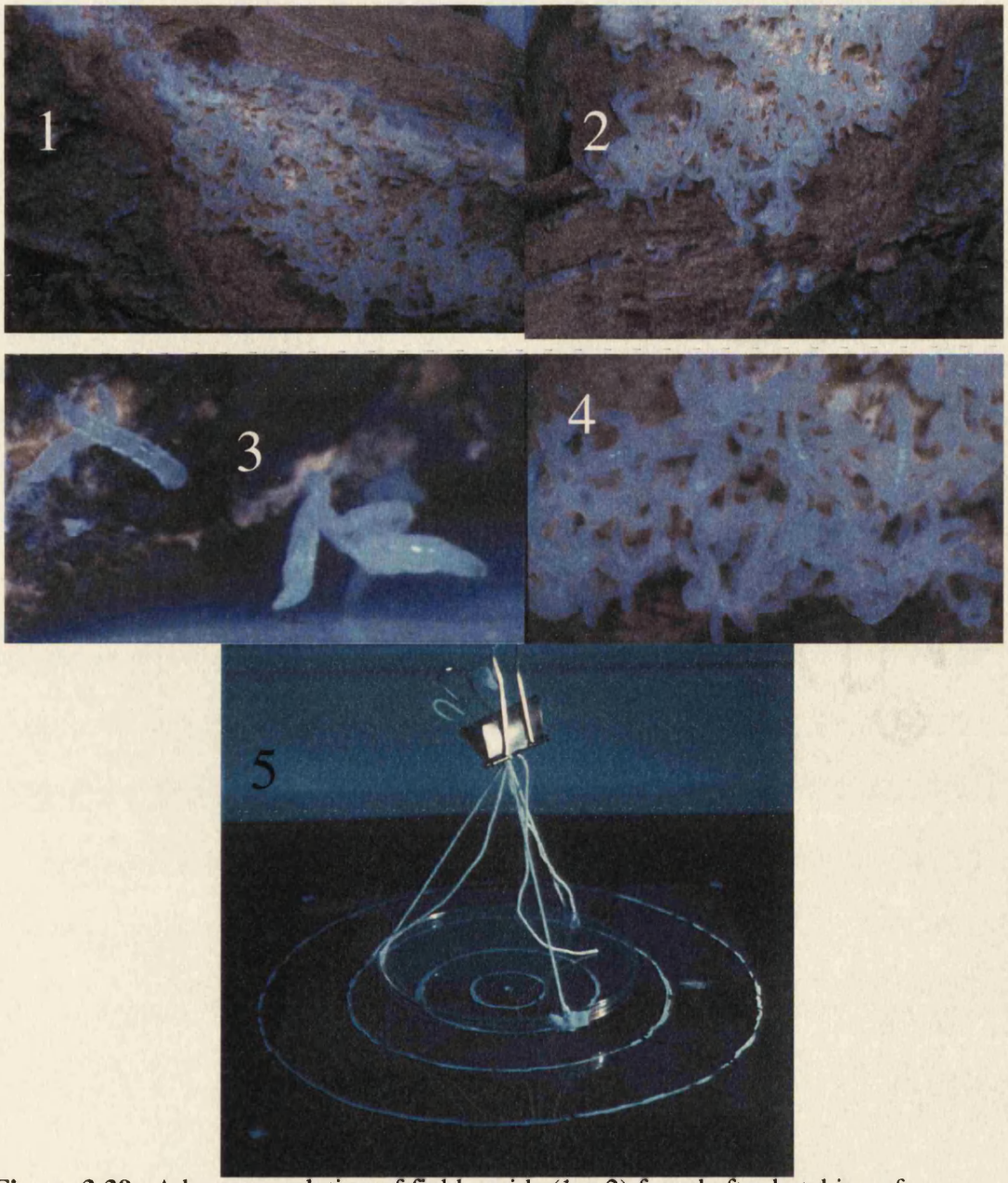


Figure 3.39 A large population of field cecids (1 + 2) found after hatching of hemipupae triggered by summer rains after an August dry spell. The bark was turgid with the pressure of so numerous a population of young newly born cecids (2); all cavities can be seen to be full of young cecids . 3; many of these young larvae display energetic "jumping" behaviour by coiling into a spring and firing themselves off from cracks in the bark or the bark surface into surrounding vegetation. Two cecids can be seen "jumping" horizontally in 3. 4; one cecid can be seen bottom right in the process of coiling in to a spring just prior to jumping. 5; an arena used for measuring height and length of cecid jumps. In this orientation it is being used to determine average height by adjusting the height of a petroleum jelly coated surface to which cecids stick if they contact the dish, where upon they can be counted easily.

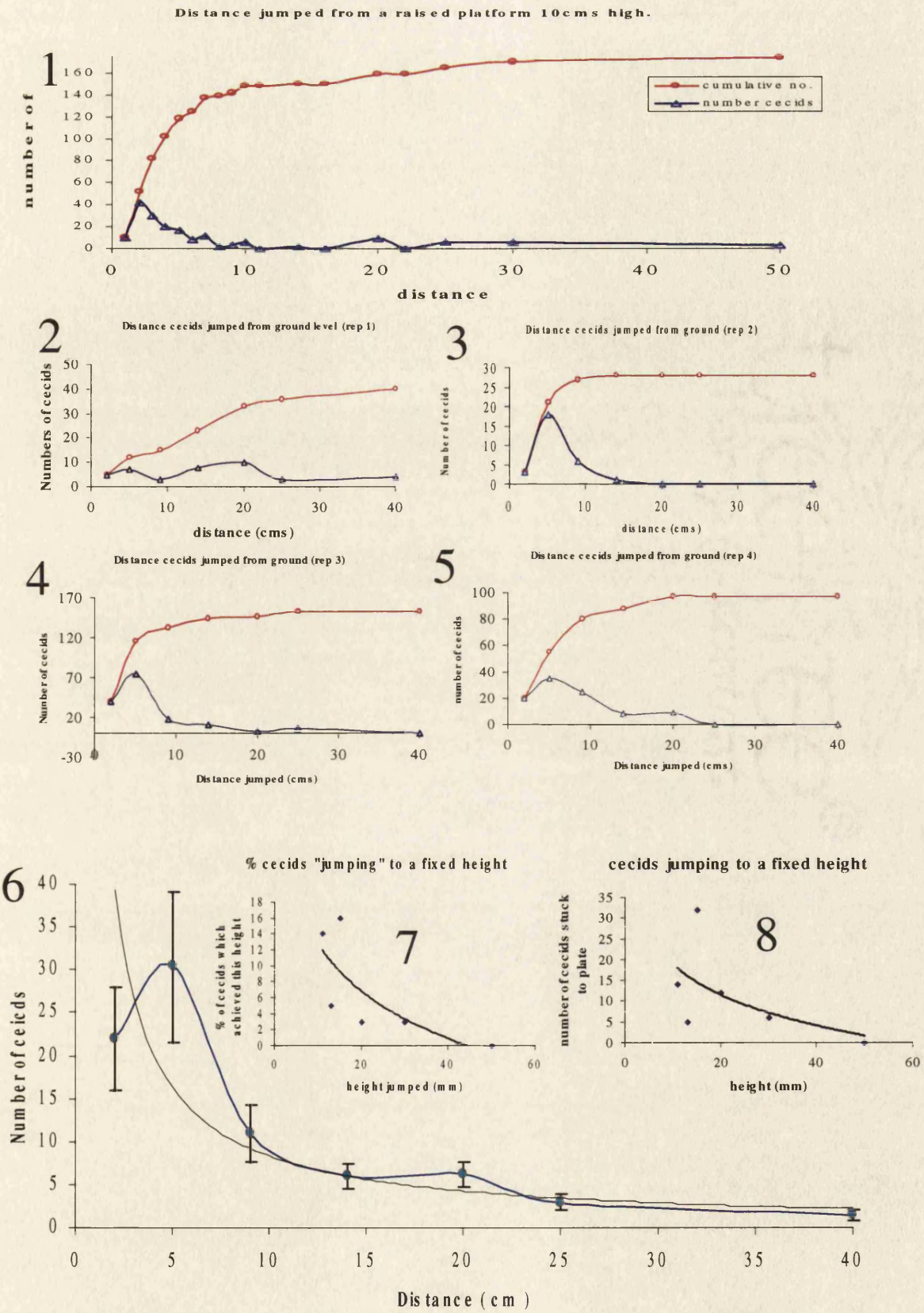


Figure 3.40 Graphs showing cecid jumping measurements; (1) shows distance jumped from a raised platform, (2 - 5) show distances jumped from 4 flat arenas, (6) shows mean distance jumped in flat arenas, (7) shows % cecids reaching heights, (8) shows total number of cecids reaching heights.

from bark placed flat in the centre of the arena. These latter graphs are incorporated into graph 6 of mean data for distance "jumped" from the flat. Height trials are shown as % of larvae reaching certain heights from the flat in graph 7, and total number of cecid larvae reaching certain heights in graph 8. An observation was made. Larvae that travelled the furthest distances followed the same trajectory through the air with a certain angle of elevation from the starting point at the centre of the arena. This must have ensured that enough height was gained to enable the lateral component of the larval vectors of momentum to continue unimpeded to the greatest distance before the larvae came to rest.

3.5 Discussion

3.5.1 Distribution patterns

Distribution patterns for different scales of viewing *Brittenia fraxinicola* and interactive *Hypoxylon fuscum* mycelium under *Corylus* bark showed different fractal dimensions. Although this seems contrary to a generally accepted view of fractal patterns which declares that, all other things being equal, such patterns show self-similarity at any scale (Gleick 1987), these results suggest that between-scale heterogeneity has an ability to break the symmetry of the distribution pattern so that it is no longer self similar at every scale (Keller 1983, Rayner 1995, Rayner 1997, Thom 1972). Fractal patterns which show self-similarity are grown at once in homogenous conditions and in selection vacuums (Rayner 1998 a), where nothing was in that niche before, so nothing is there to break pattern symmetry. However, in ecosystems, successions of pattern formations overlap each other like waves (Thom 1972, Watts 1999, Wu 1995), occurring at vastly different scales despite the biological pattern forming processes being self-similar in overall process. There is surely as much diversity of the mechanism of implementation of such biological process as there are species that form distribution patterns. There is no reason to believe that nature's fractal patterns need be the same at every scale once we accept their interactive properties and propensity to overlap asynchronously with each other in space, scale and time (Prigogine 1980, Ramsdale 1999, Ramsdale & Rayner 1996, Roos & Sabelis 1995). What these results suggest is that, when regarding an assemblage of interacting species, the mechanism of distribution process must change for each scale of view from landscape to microcosmic micrometres (Wu 1995, Taylor 1998 a). In the case of cecid-fungal relations this means that the efficiency of filling space is far greater (more heterogeneous) for fungal decay columns in a dead hazel log and individual cecid larvae in particular situations of bark layers, than it is for logs within the study site, or distributions of logs, log piles and hazel stands with their resident wood-decay fungal communities, within the landscape. This indicates that the different components of a species assemblage such *as B. fraxinicola, H. fuscum* and *Corylus avellana* have different distribution mechanisms and relate to each other at different spatial and temporal scales (Wu 1995, Hugueny & Cornell 2000). Furthermore, individual fungal colonised standing poles may be may be viewed as islands in space and time (Sverdrup, Thygeson & Midtgaard 1998, Kirby, Reid, Thomas & Goldsmith 1998). Also the mechanism for distribution at each scale is likely to be different. These formulae do not necessarily differ due to any particular set of relations above any others, since many other relationships occur at different scales for each species in an assemblage (Wu 1995). However, it does mean that each scale of interaction does have a small constitutive effect on the other, and so, amongst a sea of many other factors, cecid - fungal relations could influence fungal - hazel relations, which in turn could influence the overall pattern of dead hazel in the landscape (Taylor 1999 a, b).

3.5.2 Rapid fungal survey and log piles mapping - discussion

As expected, the physical dimensions of log piles showed no significant relationship to the fungal diversity held within them, indicating, as expected, that much of the fungal community which emerges in dead hazel wood, is seeded by latent mycelia within the dead xylem of the living tree (Boddy & Swift 1983, Boddy & Swift 1984, Rayner 1996 c, Rayner & Boddy 1988) before it undergoes transformation into a dead hazel pole or is artificially assembled into a log pile (the presence and ramification of these latent mycelia will become important in chapter 7). A high diversity of 52 fungus species with exactly half of that number with only one frequency of observation shows that a large number of rare species are supported in this habitat. A large number of rare species is a hallmark of a mature climactic community (Magurran 1988) in constant succession. If the number of species with low frequencies were proportionally much lower it could be said that the habitat was less stable and consequently less able to support rare species (Wilson 1992 a). Margalef's diversity index of 10.14 is higher than that achieved in sites of earlier studies in study plots slightly to the South of the present study area (see chapter 2). 52 fungus species compared to 5 tree species and six ground vegetation species mapped shows fungus species richness to outweigh plant species richness by 5.2:1. This is slightly lower than the ratio 6:1 predicted by Hawksworth (Hawksworth 1991) but his estimate did take into account lichen species, most of which were not mapped in this study. The abundance of *V. commedens* and *H. fuscum* indicates their prevalence as primary ruderally selected colonisers of dead *Corylus avellana* wood in this habitat. Interaction between the two seems to attract cecid activity in sub-cortical zones of hazel whilst cecids are also relatively r-selected in ecological strategy. Significant relationships between diversity of fungi, insects and mosses growing on dead hazel wood support the idea of interactivity of fungal mycelia as being important in the provision of niche space for *Brittenia fraxinicola*.

3.5.3 *Corylus* bark - discussion

It seems likely that the insulative properties of dead bark and other sub-cortical tissues are harnessed by wood-decay fungi in their development. Bark forms an ideal insulation from water stress (i.e. desiccation) and oxygen stress (a consequence of desiccation). When the function of bark has been served insulating the plant phloem, bark continues to sustain conditions for life in the insulation of other sub-cortical organisms. Such life includes relatively un-insulated and vulnerable mycelia of wood-decay fungi and symbiotic co-existent insects. Likewise, gaps in bark cover represent sites both of vulnerability to stress (and consequent need of insulation or biochemical protection via chemical PSP zone pigmentation) and also emergence of insulated exploratory and distributive fungal structures such as chords, fans, rhizomorphs and fruiting bodies. Adult stages of insect life-cycles, also more insulated than larval stages, may emerge from these sites too. In cecids it may be that the formation of adults is cued by environmental stress in much the same way as stress to fungal mycelia acts as a cue to form fruiting bodies. Indeed, **perhaps both fungal fruiting bodies and cecid adults perform the same ecological function for their respective genets, to reproduce and distribute themselves to new available niche sites as they emerge elsewhere in the forest**. The strong relationships between cecids, other insects, and deeply melanised fungal regions suggest a **biochemical factor** involved in the insect-fungal relations under bark. However this is matched by **equal evidence for physical conditions** in the form of **sub-cortical cavity dimensions. It seems that where biochemical and physical conditions are combined**, differently sized *B. fraxinicola* larvae will be found inside all cavity-sizes into which they are physically able to fit. This suggests

that the **biochemistry of fungal interactions that produce deeply pigmented PSP zones is somehow involved in the physical propensity for cavities to form.** This is likely to be mediated by the biochemistry of oxygen and or the physical and chemical properties of water (Rayner, Beeching & Watkins 1995, Rayner 1999). Some weight is added to these ideas by the relation between wet sub-cortical zones and viable as opposed to hemipupal *B. fraxinicola*. Also these findings suggest that **cecids have adapted to disperse themselves non-randomly according to cavity-size distributions in the sub-cortical zones of decaying wood.** Such **fractal dispersion** could enable cecids to **escape predation** as they themselves emerge **by being able to sub-divide and fit into cavities** in which predators cannot. Alternatively, this dispersion could be to keep up with a very ruderal process which occurs only at the very beginning of cavity formation in wood, as mediated by ruderally selected members of the fungal decay community such as *H. fuscum* and basidiomycetes such as *V. commedens* and *Hymenochaete corrugata*. It seems more likely to be as a consequence of combined biological, physical and chemical potentials that cecids disperse themselves in this way rather than due to temperature relations, for which the data was inconclusive and would need far finer scaled way of monitoring (for example a probe within a single hypha or cecid to measure the relation between chemically induced oxidative stress and the formation of heat-shock proteins) in order to fit into an overall synthesis (Worthen & Honey 1999).

3.5.4 Insect emergence trapping - discussion

Since the identification of invertebrates and insects emerging as adults from single logs was not carried out to the level of species, no biodiversity indicator could be calculated (Magurran 1988, Wilson 1992 a, Brasier 1996). However, it is clearly the case that, as expected, the insect biodiversity trapped from a single niche within this ecosystem is equivalent to the biodiversity of fungi and plants for the whole ecosystem. In other words, for such a huge profusion of insect and other invertebrate biodiversity from such small domains of forest, their efficiency of filling space must have great fractal dimension as a measure of highly efficient and heterogeneous overlapped space-filling (chapter 1.7). The accommodation of insect biodiversity on bark could thus be highly heterogeneous and of fractal quality as suggested by Edward Wilson (Wilson 1992 a). An indication of insect succession within decaying hazel is given by the differences in trends between invertebrate trappings between spring 1998 and 1999. Emergence of the smallest dipteran orders including Cecidomyiidae and Sciaridae declined over time, whereas beetle, weevil, parasitic wasp and arachnid populations increased. It remains possible that Phoridae, along with other candidate predators such as predatory mites (Enkegaard, Sardar & Brodsgaard 1997, Ydergaard, Enkegaard & Brodsgaard 1997), and nematodes (Montague & Jaenike 1985) eat cecids occasionally when the opportunity arises. The temporal trapping of cecid as compared to other invertebrate groups does not vary significantly, but in proportion, the cecid adults are very few in mumber, and in the second spring of 1999, were even fewer in proportion to other trapped taxa. This again indicates that cecids are ruderal in ecological strategy, as pioneer colonisers of decaying wood and fungi. Another difference between cecid catches and non-cecid catches was that more cecids were found on vertically orientated logs and not in log piles but in natural standing coppices. This result was the opposite of that for invertebrates as a whole. **Such results add further weight to the concept of the cecid niche as being of a particular chemical and physical nature, whose properties themselves protect cecids from predation and competition.** Still-standing dead hazel poles in a coppice provide a protected and, ephemerally distributed niche that is hard for other species to invade, at least temporally. However, cut wood placed in piles represents a different kind of niche because the exposed and cut surfaces are open to invasion from whatever is small enough to enter the cut and

exposed bark architecture. A second possibility is that the standing hazel poles were more recently created niches than log piles, so the reduced cecid trappings from log piles could be attributed to insect and fungal succession. **The fact that cecids seem highly tolerant to particular chemical environments (which may repel other inverterbrates) is supported by liquorice trapping results. The fact that no paedogenetic species of cecid was found in the traps indicates that perhaps the population of *Brittenia fraxinicola* I had chosen to investigate possesses an adult phase so rarely that sexual reproduction may have been selected out, or perhaps the traps needed to be placed for longer.**

However, the fact that no adults emerged (either from the field or from laboratory cultures) from this paedogenetically reproductive cecid larval species was one of the points of evidence that led to a positive identification. Together with the use of scanning electron microscopy, this enabled identification of the most common field cecid larvae as *Brittenia fraxinicola* on eight points of evidence based on: creeping welt spinule row numbers, anterior spinules in front of creeping welts, dorsal and ventral creeping welts on their third thoracic segments, posterior sensoria behind creeping welts, their palp shape, proportions of head and four anal lobes with six barbs (Wyatt 1967). In addition, scanning electron microscopy allowed a potential cecid pathogen to be seen (a fungus which is still to be identified), a potential *B. fraxinicola* predator (the orange cecid species still to be identified), as well as evidence on the field cecid cuticles that hyphal fragments can be carried by cecids on their creeping welts.

3.5.5 Succession, cavities and paedogenesis - discussion

The succession of cavity-sizes regarding the long term studies of logs within wood occupied by interactive fungi over time, and the physical potential to find cavities within certain dimensions of woody debris or tree species points once more to ecological succession and how this dynamic pattern of events is seeded by interactive processes between populations of organisms in ecological relation (Frankland 1992, Ponge 1991). It seems that cecids are superbly suited to a niche which is continuously forming and extending itself as a complex in which cecid larvae paedogenetically sub-divide and become capable of catching up with the fungal succession induced dimensions of cavity-size enlargements. This leaves the larger mother cecids behind as they grow too large to fit in the smaller cavity-size classes. In this way, cecids can be considered as metaphorically "surfing" as they strive to continuously wriggle into the smallest and most freshly produced "waves" of successional interactive fungal decompositional matrixes. It is here where the *B. fraxinicola* niche is being continuously formed as the pioneer interactive fungal complex migrates through the sub-cortical zone. Cavity formation could conceivably be seeded from the chemical and physical interplay between wood, interactive ruderal fungal mycelia and cecid activity (Worthen 1989 (a,b), Worthen, Jones & Jetton 1998, Worthen & Moore 1991, Ponge 1991, Frankland 1998, Ranta, Kaitala & Lundberg 1998). Such a complex of factors leading to larval niche space realisation could be described through site-dependent feedback processes previously described for *Drosophila* metapopulations (Rosenhouse, Sherry & Holmes 1997, Wertheim, Sevenster & Eijs 1997).

The decline in field cecid populations and their immobile rigidity between April and August suggests a mechanism for surviving desiccation by insulation until conditions for reproduction occur towards the autumn. That laboratory incubation of moistened rigid larvae leads to the appearance of live progeny indicates that rigidified larvae are indeed the dormant stages of a paedogenic life-cycle known as hemi-pupae

(Wyatt 1967, Wyatt 1961, Wyatt 1963). Perhaps what is true for fungi can also be said for their cecid feeders. In temperate climates the optimum season for growth and reproduction generally occurs at the time of maximum water availability in spring and autumn (Mamaev & Krivosheina 1993, Rayner 1992 a). Evidence to support the importance of water relations is provided by the phenomenal numbers of first-instar *B. fraxinicola* jumping larvae emerging from under the bark of a standing hazel pole 9 feet above ground just hours after rain which ended a mid summer dry spell in August 1999. Jumping trial results were obtained from these larvae, showing that, potentially, **such larval activity could distribute a larval population high in the canopy of a hazel coppice over a considerable range, especially in wind. In this way, such first-instar larvae can be considered as equivalent to asexually produced seeds of plants reproducing clonally to new niches** out of reach by mere vegetative propagation alone. It is highly likely that many such larvae are able to disperse to other cavities where they might find mycelia on which to feed. Much as with plant seed dispersal, it is unlikely that an individual larvae will actually find the same niche and establish a dense cecid colony like the one from which it was liberated. However, it is highly likely that it will find some mycelium in wood or bark or leaf litter and feed on it, grow and perhaps undergo a single paedogenic cycle, thus enabling its 15 or so progeny to continue to search radially outwards for cavities. **With such overlapping processes of dispersal, search, growth, sub-division and jumping, as in a relay system, it will not be long before almost all available and newly created cavities within a habitat become encountered by cecid larvae. Their method of dispersal may seem chaotic, but because of the fractal architecture, distribution, asynchronous development and consequent unpredictability in formation of their complex niche, this method of dispersal actually has an extraordinary efficiency about it. Where a more determined strategy might fail, because it misses out a local potential, the former dispersal strategy of relating to, trying-out and moving-on seems highly adaptive. Is this strategy so advantageous that it has made the cost of firstly sexual reproduction, and then producing adult stages, too costly and hence deleterious in comparison (Mogie & Hutchings 1990, Mogie 1992)?** The observation of both pale and orange field cecids suggests that there may be several cecid species available for study from this habitat although only *B. fraxinicola* has been cultured to date. Also, the observation of a totally enclosed but photosynthetically active upright of moss inside a colony of *Phlebia radiata* suggests a hypothesis that multiple opportunities for symbiotic interaction are made available whenever a colony of one species grows, as in a microbial bio-film, over a colony of another species when both are growing on a surface or substratum. It may be possible to re-interpret the importance of endosymbiont theories in the light of bio film interactions between colonies and individuals on surfaces. It was also interesting to note the association of non-cecid dipteran larval infestations of *P. radiata* with those areas where the fungus had grown over moss.

The overall result of fieldwork presented in chapters 2 and 3 is an appreciation of the spatial heterogeneity and inconstancy of the environments (Pacala & Deutschman 1995, Phillips 1997) of field cecid populations and the fungi they associate with symbiotically. Such a relativist approach in ecology (Sparrow 1999 b), as well as enriching the study of the particular species assemblage in question, produced some new and surprising insights about topics that I suggest could easily have been overlooked previously. **The following chapter 4 will take these observations and subject them to close scrutiny at the hyphal and metabolic scales of pure fungal cultures (bipartite when including cecids) and enzymatic assays.**

CHAPTER 4: LABORATORY STUDIES OF INTERACTIONS BETWEEN CECIDS AND FUNGI FROM DECOMPOSING HAZEL WOOD, I: CECIDS ON PURE FUNGAL CULTURES

4.1 Synopsis

Pure cultures of cecid and fungal species were grown to see what effects they induced on each other in the laboratory. Fungi generally reacted to cecid grazing and foraging by increasing insulated aerial mycelium and biochemical pigmentation, enhancing catalase activity, reducing H_2O_2 production, increasing antioxidant production and reducing peroxidase activity, although the degree to which these occurred varied between fungal species. High Pressure Liquid Chromatography (HPLC) spectra of fungal metabolites differed between strains and developmental states of the same as well as different fungal species. Generally, peaks at long retention times (more non-polar compounds) changed due to ageing of mycelia and also cecid treatment. Cecid larvae were sensitive to their host fungal environments and reacted by altering their foraging patterns in response to changes in hyphal insulation, mycelial growth pattern, mycelial age and biochemical pigmentation. Cecids also reacted to new fungal environments by altering their foraging patterns and the scale of their foraging ambits as well as the speed of their life-cycles and number of progeny. In these respects, development of both insects and fungi were affected by each other, thus illustrating developmental feedback processes.

4.2 Introduction

4.2.1 Response to environment

Having investigated the spatiotemporal nature of the distribution patterns of cecids and their fungal hosts in the field, experiments were carried out initially deriving from the isolation and subsequent pure culturing of the most common field cecid *Brittenia fraxinicola* and its fungal hosts. Experiments tested early hypotheses deduced from field studies and involved growing pure isolates of several fungal species from the field and then culturing them with and without cecids. Experiments involved monitoring aspects of cecid and fungal mycelial development. Cecids were presented with different fungal environments in which to forage. Different fungi were monitored in terms of mycelial development with and without treatments of cecid larvae. Broadly speaking the experiments sought to answer two basic questions. **Firstly, what are the effects of differing fungal environments on cecid larval development? Secondly, what are the effects of cecid larvae on the development of fungal mycelia?** Early experiments also used laboratory strains of two cecid species *Mycophila speyerii* and *Heteropeza pygmaea,* which had originally been isolated from local mushroom beds and used by Justine Hunt in her work on the influence of diet-derived hormones (derived from sterols found richly in fungal protoplasm) and other environmental conditions on the paedogenetic cycle (Hunt 1996). The work on cecid paedogenesis in response to environment is thus meant to shed light on cecid developmental biology in general, rather than specifically to *Brittenia fraxinicola*, which at the time of these experiments on *M. speyeri* and *H. pygmaea*, had not yet been discovered in the local field site.

4.2.2 Effects of fungi on cecid development – introduction

Stages in the life-cycles of cecid cultures of *B. fraxinicola*, *H. pygmaea* and *M. speyeri* can be seen in figures 4.1 to 4.4 as well as some of the ways in which they interact with regions of their fungal hosts. Experiments in this chapter focus on mycophagous cecid larval paedogenic life-cycles and the two ways in which data has been derived from them, firstly in the form of the length of time taken from paedogenic birth to paedogenic birth, called generation time (Gt), and secondly in the number of first-instar larvae hatching from their mother at birth, called progeny number (Gno). Related to these two facets of paedogenesis are the size of mothers before giving birth, the size of her offspring at birth and the entrance or exit from dormancy in the form of hemi-pupae, or the formation of real pupae from which adults might emerge. An early hypothesis to test was: "That species of paedogenically reproducing cecid adjust the speed of their life-cycle development according to their fungal environment". The aim of life-cycle experiments was thus to test the null hypothesis: "That a species of cecid would not show significant growth rate differences, either in terms of speed of larval cycles or number of larvae born from each mother, when cultured in different fungal environments." Another developmental feature from which to derive data were the pathways or trajectories left behind by larval foraging, in terms of their position, thickness and curvature.

4.2.3 Effects of cecids on fungal development – introduction

Figure 4.5 shows *H. fuscum* fungal cultures with different degrees of melanisation with and without *B. fraxinicola* cecid larval treatments. Experiments in this chapter focus on the degree of pigmentation of the ascomycete *H. fuscum* and growth of thickly insulated aerial mycelium by the basidiomycete *V. commedens* as introduced in chapter 1. At a biochemical scale the experiments seek to relate the aforementioned developments to possible build up of oxygen related stress, reactive oxygen intermediates produced in hyphae as they age or senesce in response to increased oxidative stress (see chapter 1). Changes in colour and morphology of mycelia and the production of chemical antioxidants and enzymes which reduce the formation of reactive oxygen species by removing one of their main inducers, hydrogen peroxide (H_2O_2), such as catalase and peroxidase, and the production of hydrogen peroxide itself, are all investigated in an attempt to build a synthesis. This synthesis relates results of these single culture laboratory experiments to oxidative stress and its remedial metabolism in both fungi and insects.

4.2.4 Tissue printing & metabolite profile analysis – introduction

A novel strategy and method were developed to determine the distribution of enzyme, oxidant and antioxidant activity in fungal mycelia with and without the influence of cecids. As far as I know, tissue printing has not yet been used for fungal biochemical assays but has been used for plant and animal tissues (Schopfer 1994). The technique used here was developed with the help of Kim Reilly (cassava plant molecular biology research group) here in the Department of Biology and Biochemistry, University of Bath. Ms. Reilly has been using and developing her own techniques of tissue printing to look at oxidative stress related chemical and enzyme activity in senescing and physiologically deteriorating root tubers of the African and South American staple cassava (*Manihot esculenta* Cranz). Essentially plant and animal tissue printing methods involve absorbing proteins and other metabolites into the pores of a nylon or nitrocellulose membrane, where they become fixed in position by electrostatic charge. The membrane prints are then transferred into various developing reagents, which stain for particular reactions to show the location and quantity of substance being investigated. The results are both qualitative and semi-quantitative, showing the

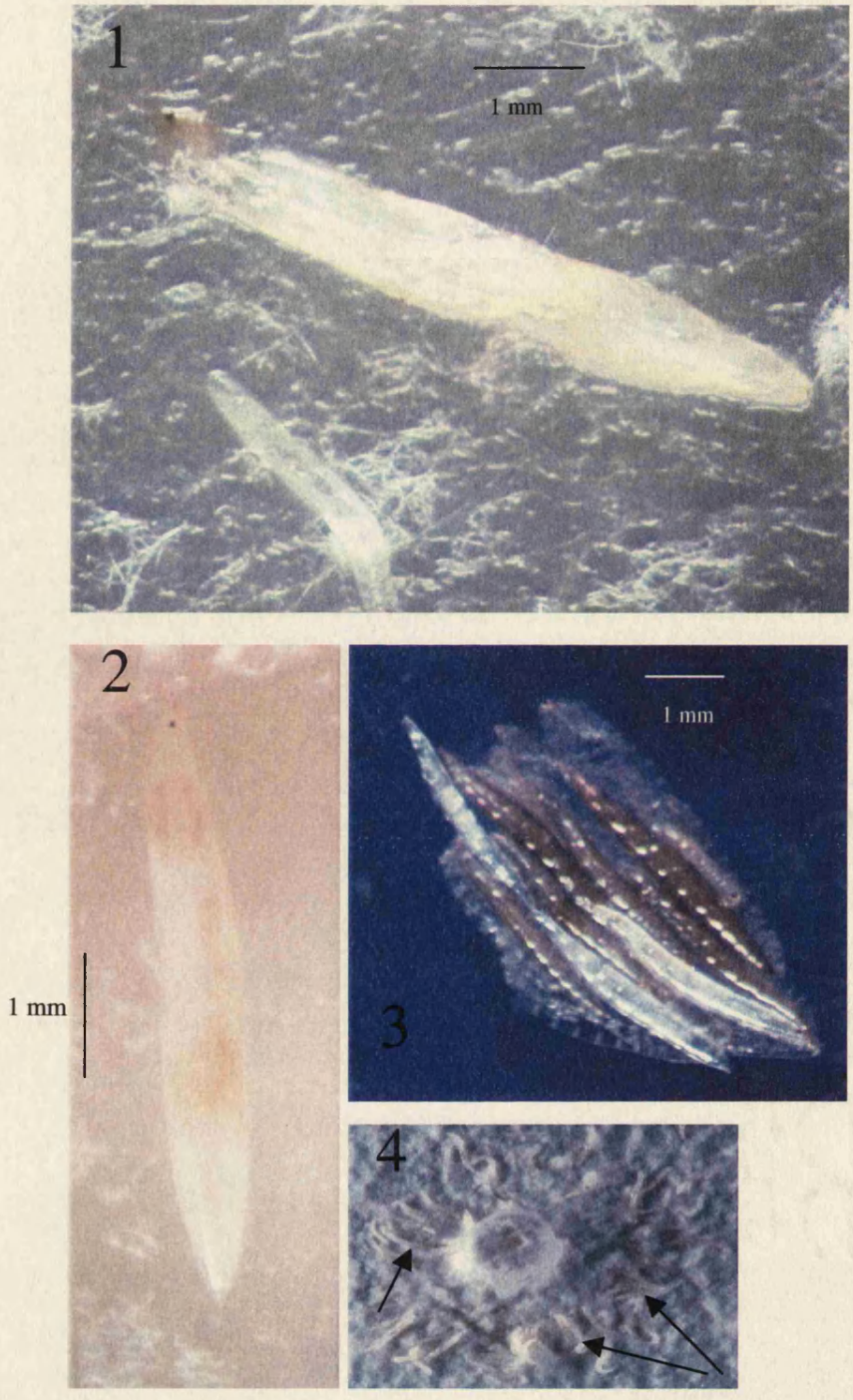


Figure 4.1 field cecids in pure fungal cultures of *Chondrostereum purpureum* . 1; a mother cecid lies dead next to the first of her daughters to emerge after paedogenic birth. 2; a mother before birth, 3; hemipupae which can survive dessication, 4 a cluster of young (arrowed) around the fungal inoculation plug.

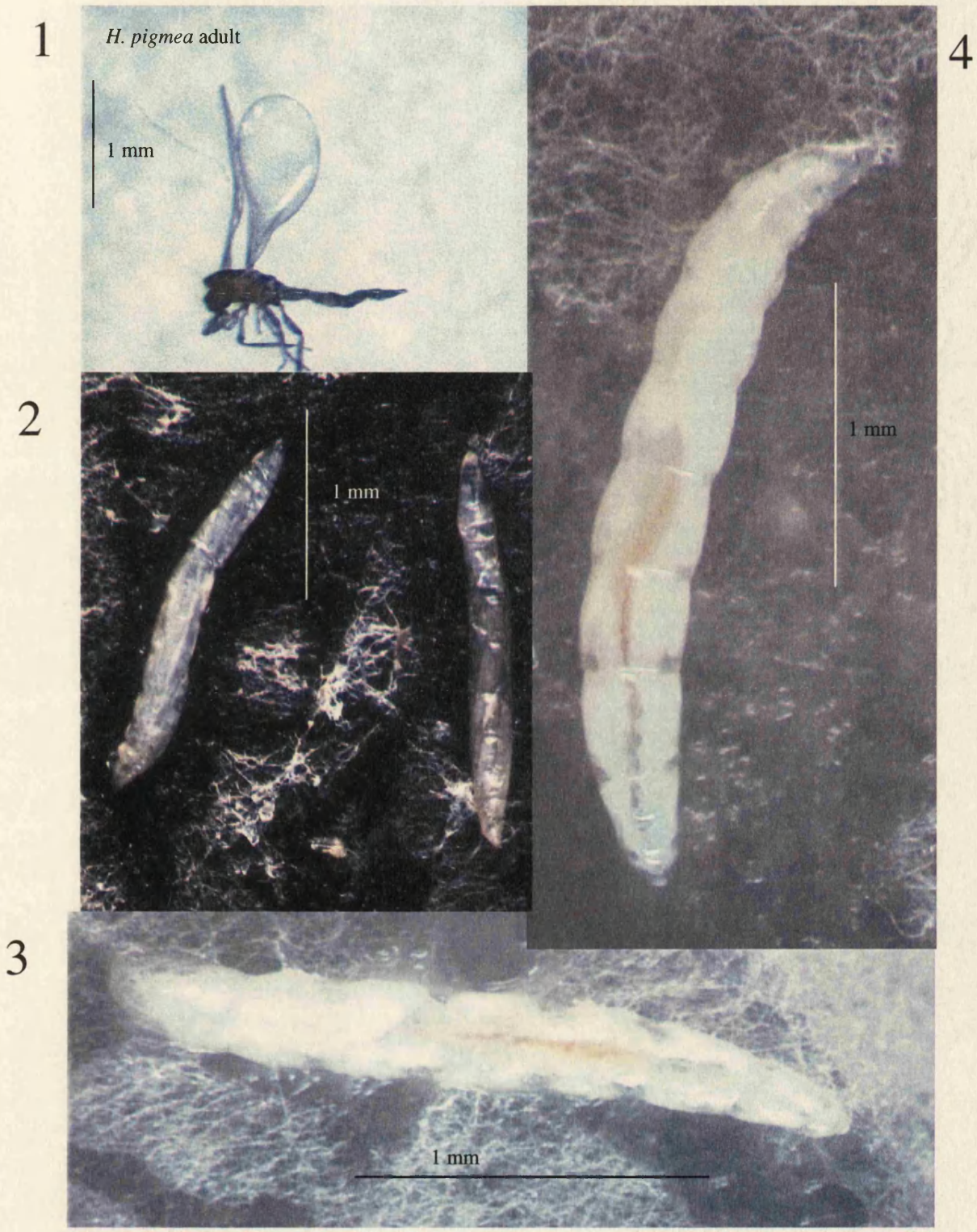


Figure 4.2 Magnified images of the cecid species *Heteropaeza pigmea*. (1); a rare imago, (2) two mother larvae lie on a deeply melanised region of ascomycete *H. fuscum* mycelium. They are stacked full of daughter larvae inside and are about to give birth. (3) and (4) ; juvenile larvae cultured on *C. purpureum* with white fatty tissue packed around discoloured guts.

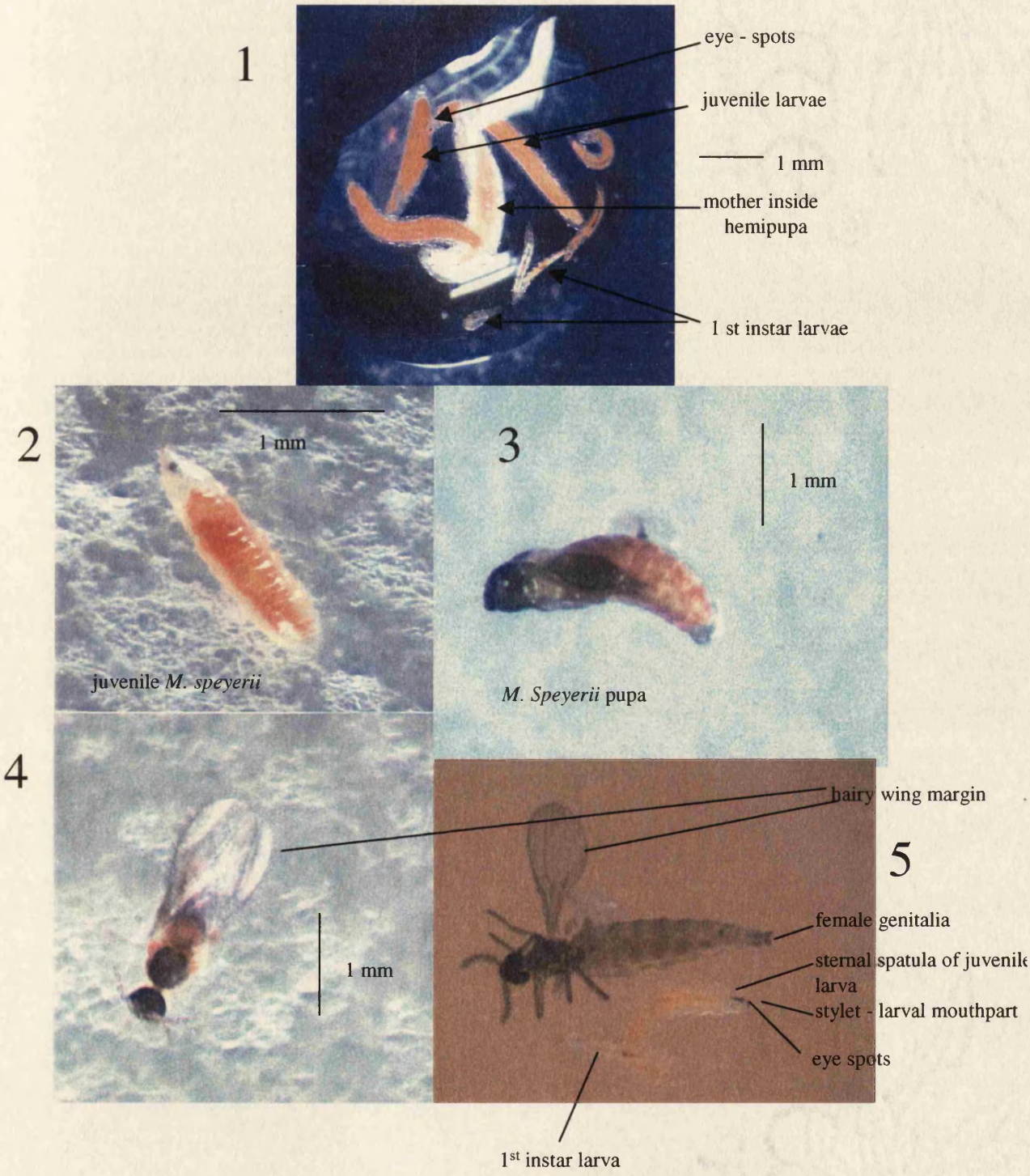


Figure 4.3 Stages in the life cycle of the cecid species *Mycophila speyerii.* (1) a drop of water holds a variety of sizes of larvae from newly born to mother, (2) a juvenile larva forages through mycelium of the fungus *Chondrostereum purpureum.* (3) a *M. spererii* pupa. (5) an adult with two larvae; one juvenile and one 1st instar.(4) an adult recently emerged from pupal chamber on *C. purpureum* laboratory culture.

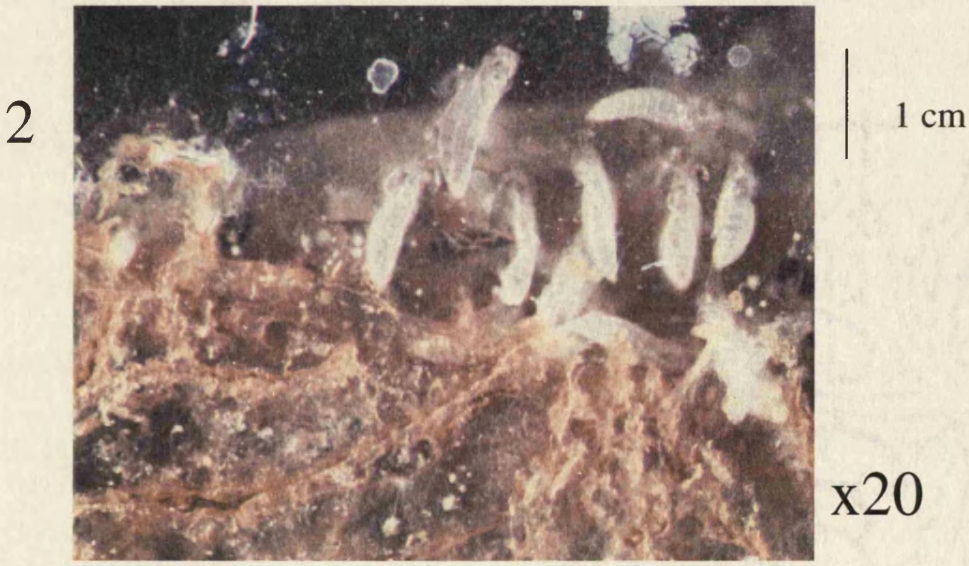


Figure 4.4 (1); Young new-born cecid larvae at *V. commedens* colony margin one day after paedogenesis from a single mother. These are *Mycophila speyerii* larvae. (1) and (2) show cecid development on pure cultures; (1) shows young *Mycophila speyerii* larvae foraging rapidly on young, thick, lipid rich hyphae of *V. commedens* margin. (2) shows older *M. speyerii* larval pupae in an older culture *V. commedens* . (2) ; edges of old *V. commedens* cultures, where agar rolls and or lifts; sometimes into corrugations, *Mycophila speyerii* hemipupae and genuine pupae would often be observed. Here ecdysed eppidemal pupal cuticules left from imago alate emergence can be seen.

H. fuscum Strain one

H. fuscum Strain two

Without cecids

With cecids

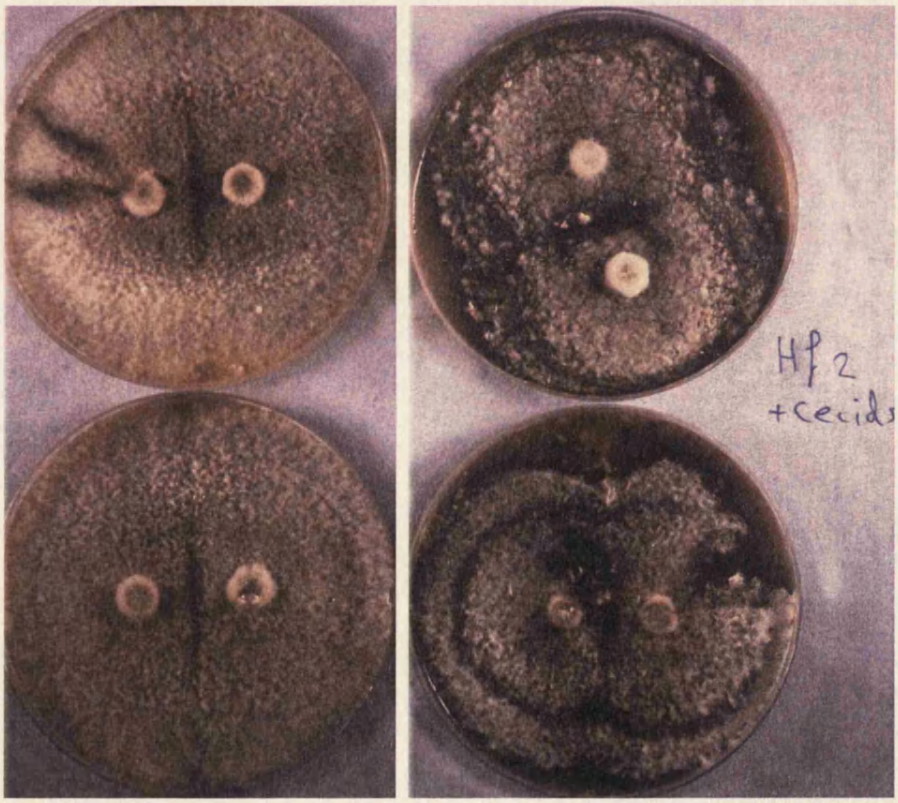


Figure 4.5 Pure *Hypoxylon fuscum* cultures; at top the difference between field isolates stain 1 and 2; both of which were obtained from isolated from field logs in which cecid populations had been found. Below are the results of a typical experiment with half of the cultures subjected to treatment with *B. fraxinicola* larvae.

locations of activities as well as amounts - each print becoming a map of the biochemical activity in question.

Tissue printing on nitrocellulose membranes is used (adapted from Schopfer 1994) as a novel method for investigating oxidative biochemistry inside fungal mycelia. Results from tissue print localisation of reactive oxygen intermediate metabolism are compared with changes in High Pressure Liquid Chromatography (HPLC) elution spectra, especially at non-polar end of the solvent gradient where water insoluble double-bonded phenolic molecules appear. **Catalase activity, thought to be harnessed particularly by chemically versatile Ascomycetes, is usually confined to peroxisomes and glyoxisomes within the hyphal protoplasm where the enzyme turns excess H_2O_2 into water and oxygen, or, when not in excess, uses H_20_2 to mediate "peroxidative" oxidation reactions, sometimes in place of normal metabolic oxidation reactions, from phenols, formic acids, fomaldehydes and alcohols into their oxidised and less toxic forms** (Alberts *et al.* 1989). On the other hand, **fungal peroxidases, thought to be harnessed particularly by hyphal boundary dextrous Basidiomycetes, are a diverse family of iso-enzyme complexes, usually found extracellularly where they help break down lignin and other molecules into phenolic aromatics via a reversal of the Shikimate pathway**. Peroxidase action releases intermediary free radicals in the process, for example from coumaryl-alcohol to coumaryl - Co A as a step leading eventually back to phenylalanine as a lignin precursor molecule (Anderson & Beardall 1991).

High Pressure Liquid Chromatography (HPLC) is a method widely used for separation and quantification of bio-molecules in complex mixtures. As such, it is an important tool for the investigation of secondary metabolite biochemistry in fungi (Crowe 1997, Watkins 1998, Patterson & Bridge 1994). HPLC elution metabolite profiles lack a scale on their vertical axis because the profiles represent a qualitative measure of UV absorbance relative to other peaks, and not a quantitative test. Retention times of peaks produced by eluted compounds can be compared only when using the same solvent gradient system. This is because subtle changes in solvent gradient system produce fundamental changes in retention times and even the order in which some elutants appear. However, at a more basic scale of HPLC elution spectra comparison, general trends of elution spectra polarity can be compared, even between solvent gradient systems. This basic, coarse-grained but easy method of qualitative comparison uses the principle that, at the beginning of the elution spectra, the first chemicals to be eluted are polar molecules, which easily dissolve in the water-based buffer. These peaks appear at the beginning left hand side of the spectra between five and 14 minutes. As time progresses, we see peaks produced towards the right with longer retention times which have a more non-polar nature and are consequently removed from the HPLC column when it is eluted with 100 % acetonitrile, a very non-polar solvent. By looking at and comparing the general trends in proportions of non-polar and polar metabolites, we can gain a basic understanding of the type of biochemistry that is going on to produce these changes. This understanding relates the production of oxidative stress that might be attributed to ageing or cecid behaviour, to mechanisms within the fungal species to cope with increased reactive oxygen intermediates (ROI). Such mechanisms, as suggested in chapter 1, and shown already in this chapter, cause the Ascomycotina (such as *H. fuscum*) to produce protoplasmic antioxidants, and cause the Basidiomycotina (such as *V. commedens*) to produce increased hyphal insulates. Antioxidants are usually more polar in their solubility and dissolve readily in water-based buffer, whereas hyphal insulates are generally composed of hydrophobic phenolic residues that become sequestered into a lattice arrangement

during hyphal wall synthesis (Burnett 1979). Changes in the proportions of production of polar and non-polar metabolites will be detected by HPLC analysis. So, we could expect to see some general shape changes in HPLC profiles as ascomycete and basidiomycete fungi become physiologically "stressed" by ageing or cecid treatments. Consequently what I expected to see was that such changes would cause opposite trends in metabolite profiles for *H. fuscum* and *V. commedens*. It was expected that the ascomycete profile of *H. fuscum* would show an increased number of peaks at the left hand end, in response to increased polar metabolite production, and the basidiomycete profile for *V. commedens* to show an increased number of peaks at the right hand end, in response to non-polar compound production. These changes can best be qualified by visualising a curve of best fit through the data for each wavelength's plot. This simplifies the complex profiles to simple mean distributions. Unfortunately it is difficult to export Gilson data to compatible software that can compute these general trends since this use for HPLC profiles is somewhat unorthodox. Fortunately, these general trends are easy to see without having to compute them.

4.2.5 Aims of chapter 4

Simple, linear experiments must be carried out with isolated factors before a more complex synthesis can ever be built up. The purpose of this chapter is to present results of investigations of the simplest kind - carried out in the full spirit of reductionist principles. The aims are therefore to culture, and experiment with single fungal cultures with and without treatments of cecid larvae in bipartite interactions to determine any developmental effects of cecids on fungi and *vice versa*.

4.3 Materials and methods

4.3.1 Culture systems

This section outlines methods to capture, isolate and maintain fungal and cecid species found in the field using methods adapted from Gabrischevsky (1928), Wyatt (1969), Camenzind (1963), Hunt (1997), Williams (1982), Crowe (1997), Ramsdale (1996), Watkins (1998) and Rayner & Boddy (1998).

4.3.1.1 Methods for the isolation of fungi

Fungus species with which cecids were found associated in the field were removed from plots by taking fruiting bodies or sections of woody debris in which the fungi were growing in bags and glass or plastic vials. Different methods were then used to isolate heterokaryons or homokaryons from the samples. The method tried first with all species was to try to sterilise a very small amount of mycelial tissue from within a fruiting body or a piece of wood by first breaking open the sample to expose hitherto untouched, unexposed mycelium and, with a fine pair of flame sterilised tweezers, taking a small tuft of mycelium to dip for one minute in 1 % bleach solution before putting it onto 2 % malt agar (MA) Petri dishes which had been made by adding one litre of distilled water to 20 g malt extract and 20 g tissue culture agar in a litre flask, stirring and autoclaving. Before pouring the plates, 0.01 g of Novobiocin was dissolved in 3 cm^3 distilled water and filter-sterilised by syringing through a sterile Whatman filter into the media (Watkins 1998, Crowe 1997). A second method of isolation was to pass a small tuft of the mycelium rapidly twice through the base of a blue Bunsen flame, instead of using bleach, before embedding it in Petri dish media (Crowe 1997, Williams 1982). After 24 hours, those plates contaminated were discarded and the rest observed after 48 and 72 hours for outgrowth of emergent mycelium.

Subcultures were made as soon as possible from these outgrowths by removing hyphal tips onto new Petri dishes. Two methods were used for this. Firstly, under medium power magnification light microscopy a flame-sterilised scalpel blade was used to cut through the edge of the fungal colony margin. The cut was made such as to remove a small piece of agar with only a few hyphal tips with which to start the new colony. The second method was to use an adapted sterilised microscope lens plug cutter that could cut a 1 mm^2 plug of agar in which a single hyphal tip had been selected using a normal high power objective. The cut plug was then lifted out using a flame-sterilised tungsten filament and placed to grow on fresh media. After re-growth the identification of the fungus was then checked using keys for hyphal features in culture specific to the species under high power magnification light microscopy. Features included clamp connections, and lipid droplets as well as branching patterns, hyphal dimensions and colour and morphology of colony centres as well as margins. Status as homo- or heterokaryon was determined by searching for clamp connections since only heterokaryotic hyphae produce them. Since it is improbable that a homokaryon will be isolated from a domain of woody debris and impossible from a fruiting body (unless from spores - see below), the detection of clamp connections was important to determine basidiomycete identity (Watkins 1998, Crowe 1997).

A second technique was to isolate and germinate homokaryon spores from fruiting bodies. Fruiting bodies were placed upside down in an empty Petri dish, left overnight in a humid environment by placing in a plastic bag with wet paper towelling, and the following day collected in a pipette tip with autoclaved, distilled water by repeated sucks of the water up into the pipette. A serial dilution series was made using the suspended spores and the concentration of spores in the first dilution found by using a blood haemocytometer (Watkins 1998, Ramsdale 1996). The dilution at which there would be about 20-100 spores per ml was then selected and 1 ml of dilution spread over a fresh 2 % MA Petri dish (as above) using an alcohol and flame-sterilised glass spreader (Rayner & Boddy 1998). After 24 hours, single germinating spores were selected using high power magnification and the sterile microscope lens plug cutter used to cut a 1 mm^2 plug of agar around them or to take a single hyphal tip from a germling homokaryon mycelium (Ramsdale 1996). The small colony was then transferred onto new media to continue growth. The aforementioned methods of checking fungus identity were then used.

4.3.1.2 Fungal culturing methods

Fungal cultures were routinely checked and sub-cultured onto fresh media every two weeks by taking a 5 mm^2 plug of mycelium out of the old culture, using aseptic technique with a blue Bunsen flame, a stainless steel hole borer and thin tungsten filament on a handle. Plugs were cut with the borer and then individually transferred it to the centre of labelled fresh 9 cm diameter 2 % MA Petri dishes (without Novobiocin added to the liquid media before pouring Petri dishes). The Petri dishes containing both new and old cultures were stacked upside down according to species in an incubator, which maintained a temperature of 27 °C in darkness (Watkins 1998, Ramsdale 1996, Crowe 1997). The following fungal species were cultured every two weeks: *Hypoxylon fuscum* (Hf) (Hf isolates 1 and 2), *Vuilleminia commedens* (Vc), *Corticium evolvens* (Ce), *Chondrosterum purpureum* (Cp), *Hymenochaete corrugata* (Hc), *Diatripella favacea* (Df), *Phlebia radiata* (Pr), *Phellinus ferreus* (Pf), *Encoelia furfuracea* (Ef), *Tremella mesenterica* (Tm), *Tremella foliacea* (Tf), *Bjerkandera adusta* (Ba), *Mycoacia uda* (Mu), *Phanerochaete velutina* (Pv), *Phallus impudicus* (Pi), *Coriolus versicolor* (Cv), *Stereum hirsutum* (Sh) and *Stereum gausipatum* (Sg). *Hypoxylon fuscum* 1 was a homokaryon from spore isolate whereas Hf 2 was a heterokaryon isolated from wood.

4.3.1.3 Obtaining cecid isolates method

Cecid larval stages were removed from wood by cutting or teasing away some of the sub-cortical zone in which they were present in the field and placing in a small corked glass vial to prevent them from being squashed on the way back to the laboratory. There, larvae were carefully removed from sub-cortical zone wood and frass using a fine artist's paint brush, washed for 20 seconds in 70 % alcohol, one minute in 1 % formaldehyde then rinsed in sterilised water, before being placed at the margin of a week-old culture of the fungus *Corticium evolvens* covering 9 cm Petri dishes containing 2 % MA, and stored in an incubator at 27 °C (Hunt 1996, Wyatt 1969, Camenzind 1963, Gabritschevsky 1928). After one week the cecids were placed onto new *C. evolvens* plates and kept in the incubator. All plates with cecids were wrapped in parafilm around the edge to prevent desiccation and escape of first-instar larvae. Plates treated with cecid larvae were kept below any fungal cultures. The cecid species isolated was *Brittenia fraxinicola* (chapter 3).

4.3.1.4 Cecid culturing methods

Between three to 10 larvae were removed from old cecid cultures after two to three weeks of continuous colony reproduction at 27 °C in darkness, and placed with a fine artist's paint brush wetted in 70 % alcohol and dabbed on laboratory towelling, onto the margin of new fungal colonies of *C. evolvens*. Plates were labelled with cecid species and origin and wrapped in parafilm and stacked upside down (as recommended by Wyatt 1969 and Ulrich 1934). Records were kept as to where the cecids had been located in the field; their colour, size and relative levels of activity in laboratory culture were noted. All plates with cecids were wrapped in parafilm around the edge to prevent the escape of first-instar larvae. Cecid cultures were kept as far away from fungal cultures as possible but under identical conditions of 27 °C in complete darkness. When large experiments were under way, and when there were several cecid species in culture, this necessitated two identical incubators running at the same temperature in complete darkness, one for experiments and one for maintaining routine cultures. The cecid species cultured were *Mycophila speyeri, Heteropeza pygmaea* and *Brittenia fraxinicola.*

4.3.2 Effects of fungi on cecid life-cycle development method

This experiment, carried out in late 1995 with Justine Hunt's laboratory strain of *Mycophila speyeri*, tested the hypothesis that a paedogenic cecid population would not alter Gt or Gno rates of paedogenic population growth with changes in mycelial environment. This experiment was carried out before I had discovered the source habitat characteristics or the field cecid *Brittenia fraxinicola* that fits in them (at that time *B. fraxinicola* remained un-identified). This experiment thus uses *M. speyeri*, rather than *B. fraxinicola*, as the case study for cecids in general

One *Mycophila speyeri* larva was taken from culture on the basidiomycete *Chondrostereum purpureum* , a standard cecid culture fungus due to its high lipid content (Hunt 1996), and placed on each of four one week old fungus cultures consisting of three basidiomycetes *Vuilleminia commedens*, *Chondrostereum purpureum* and *Corticium evolvens*, and the ascomycete *Hypoxylon fuscum*. Each week new fungus cultures were sub-cultured in preparation for the following week's inoculation with either one of the newly hatched cecids from the previous larval cycle, or, if the mother had not undergone paedogenic reproduction, she was transferred to a new source of mycelial nutrition. Thus, each week, cecids were transferred to new fungus plates of their test fungus and new plates were sub-cultured with each fungal host. After an initial week to let the cecid

mothers adjust to feeding on the test species of fungi, the experiment was started and results kept for six weeks on the larval cycle time known as generation time (GT), i.e. days taken from hatch of young larvae until subsequent birth of new live young, and also the numbers of young produced by each birth, known as progeny number (Gno). Any other interesting observations were also noted during this period. All plates with cecids were wrapped in parafilm around the edge to prevent the escape of first-instar larvae.

4.3.3 Effects of fungi on development of cecid foraging behaviour methods

Positions of larval trajectories were noted and measurements made of their curvature in terms of radius by stretching a compass until it could turn around an arc that coincided with the circumference of a circle that matched the shape of the part of a curve in the pathway being measured. The compass was then transferred to a ruler and the measurement noted as toroidal radius in mm (Stephens & Krebs 1986). Another method, this time to assess degree of curvature of a trajectory, was to compare the direct path distance in mm between two points chosen randomly with the actual path distance in mm between the same two points. The ratio between the two is called the ratio of curvature, with higher values designating a greater amount of curvature to the trajectory.

4.3.4 Repli-plate fungal choice experiment method

A repli-plate consisting of 25 separate wells was filled with 2 % MA to a depth of 0.5 cm and inoculated with a range of fungal species, one in each well. After one week, 1 cecid *Brittenia fraxinicola* larva was inoculated into the centre of each hyphae-filled well. Numbers of cecids in each mycelium was then monitored over time as in a choice chamber, except measuring a combination of survival, reproductive success and migration rather than "choice".

4.3.5. Effects of cecids on development of monoculture fungal mycelia

4.3.5.1 Methods for estimation of rate of ascomycete pigmentation

Eight *Hypoxylon fuscum* cultures were initiated on 2 % MA in 9 cm diameter Petri dishes by inoculating two 5 mm diameter plugs from standard *H. fuscum* isolate plates into the centre of each plate and 4 cm apart. After two weeks, by which time the ascomycete mycelia had grown out radially from inoculum plugs, fused, and nearly reached the edge of the Petri dishes, 4 of the cultures were then treated with *B. fraxinicola* larvae by inoculating 5 cecids into the colony margin with a fine artist's paint brush. The cecid larvae had previously been raised on *H. fuscum* fungal culture for one week under standard conditions. Starting on the day when cecids were inoculated, and once a week thereafter for forty days, the amount of unpigmented and pigmented *Hypoxylon fuscum* mycelium was quantified using acetate sheet and marker pens whilst holding Petri dishes up to the light to distinguish the regions more clearly, cutting out and weighing the different regions, and finally converting from weight to area using a standard 1 cm^2. Later experiments quantified pigmented areas more rapidly using Quantity One Bio-rad image analysis software volume contour-mapping tool with additional volume analysis report window (Donnelly, Boddy & Wilkins 1999).

4.3.5.2 Methods to determine effect of cecids on basidiomycete insulation

Eight *Vuilleminia commedens* cultures were initiated on 2 % malt extract agar (MA) in 9 cm diameter Petri dishes by inoculating two 5 mm diameter plugs from standard *V. commedens* isolate plates into the centre of

each plate and 4 cm apart. After one week, by which time the basidiomycete mycelia had grown out radially from inoculum plugs, fused, and nearly reached the edge of the Petri dishes, 4 of the cultures were then treated with *B. fraxinicola* larvae by inoculating 5 cecids into the colony margin with a fine artist's paint brush. The cecids had previously been raised on *V. commedens* fungal culture for one week under standard conditions. Starting on the day when cecids were inoculated, and once a week thereafter for forty days, the amount of thickly insulated aerial as opposed to thinly insulated and diffuse non aerial *V. commedens* mycelia were quantified using acetate sheet and marker pens whilst holding Petri dishes up to the light to distinguish the regions more clearly, cutting out and weighing the different regions, and finally converting from weight to area using a standard 1 cm^2. Later experiments quantified insulated areas more rapidly using Quantity One Bio-rad image analysis software volume contour-mapping tool with additional volume analysis report window. To make sure that membrane used in tissue-lysis printing (see below) did not alter significantly the outcome of fungal responses to cecid larval activities, the methods above were run in parallel with membrane controls. Sterile nitrocellulose membranes were placed on the agar surface at the time of inoculation.

4.3.6 Metabolic scale methods

4.3.6.1 Tissue-lysis printing methods

This novel strategy adapted for fungal mycelia was to combine tissue printing with hyphal lysis. This was done by growing mycelium through a sterile piece of Electran nylon membrane with pore size of 0.45μm through which fungal hyphae could grow, and then ripping off the membrane and creating the first of several prints directly from lysed hyphal contents. Electran membranes cut to 9 cm Petri dish diameter were placed onto the surface of 2 % MA Petri dishes just prior to aseptic fungal inoculation in the way already described. All printing membranes apart from the first Electran nylon "lysis membrane" were standard Hybond C Amersham nitrocellulose membranes specially formulated for protein absorption. The reason the first "lysis membrane" had to be Electran nylon was that this membrane was much cheaper, was unaffected by autoclaving, and the pore sizes of nitrocellulose Hybond C membrane were thought too small for fungal hyphae to grow through. As controls to make sure that the responses of cecids to fungi and *vice versa* were not altered by the presence of the nitro cellulose or nylon membrane, experiments of fungal pigmentation and insulation rates, areas and larval foraging behaviour were run both with and without membranes.

After running experiments for three weeks with and without cecid treatments, image analysis (as described above) was carried out on states of *H. fuscum* pigmentation and *V. commedens* insulation to test whether the presence of membranes altered previous findings. The entire membrane was then slowly ripped off allowing protoplasm from lysed hyphae to spill out simultaneously over the membrane and the agar surface, thus at once being fixed into the membrane and also forming a pool of hyphal protoplasm from which subsequent prints could be made on the exposed agar.

An advantage of the novel technique developed here is that it circumvents some problems that many researchers have faced trying to investigate localised biochemistry within sealed and insulated living hyphae. Without lysing hyphae, only the extra-cellular enzymes and a few chemicals which fungi release into their environment to digest their food are available for investigation (Paterson & Bridge 1994). However, lysed hyphal contents often degrade if the reactives are not soon fixed in place or used in an assay (Schopfer 1994,

White & Boddy 1992). Thus making a map of the distribution of biochemical activity in a 9 cm culture by removing and individually testing many small samples can be very time-consuming (White & Boddy 1992). Tissue printing had possibly not been used before because highly insulated aerial mycelium blocks absorption of protein or compounds from the biochemical activity underneath, and scraping aside aerial mycelium mixes up biochemical localisation patterns subsequently obtained. On the other hand, the combination of simultaneous hyphal lysis with the formation of the first of several tissue prints has multiple advantages as a method to investigate local fungal biochemistry within a mycelium. These are that the lysis caused by lifting the membrane from the culture removes aerial mycelium simultaneously creating a fixed print of protoplasmic constituents and inter-hyphal reactants that underlie insulated aerial mycelium.

With freshly exposed and lysed hyphae on the Petri dish, up to 5 more tissue prints were made by absorbing protoplasm with nitro cellulose membrane cut to 9 cm Petri dish diameter and pressing onto the culture agar surface with a Petri dish diameter container filled with water to provide weight. The printing time was 1 minute for each of the number of prints that had been carried out, not including the original Electran membrane used during lysis. So the fifth print using nitro-cellulose membrane (sixth print including the electran nylon "lysis membrane") was left for 5 minutes to absorb lysing hyphal protoplasm *in situ*. Marks were made on membranes and Petri dishes for orientation purposes. In two of the tests for H_2O_2 production and catalase activity, nitro-cellulose membranes had to be pre-prepared and impregnated with specific reagents before printing (as described below). To prevent possible cross-contamination of assay reagents left on the hyphal-agar surface, these particular two assays were performed at the end with catalase activity followed finally by the H_2O_2 assay. Each membrane, including the first Electran nylon membrane, was used in assays since the lysed and fixed protoplasmic contents were a precious source of information. It was important to prepare all developing reagents freshly beforehand and be organised whilst conducting assays so that whilst one print was developing, the next to be developed was being printed and so on. Membranes were handled with forceps and gloves were changed (but re-used) between assays to prevent biochemicals from one assay from contaminating results of the next. Results were photographed as quickly as possible after the developing reactions had taken place.

4.3.6.1.1 Antioxidant assay using DPPH tissue print method

The strategy of the antioxidant chemical assay, modified from Takao *et al.* (1994), was to use the pink - purple coloured free radical compound 1,1-diphenyl-2-picrylhydrazyl (DPPH), a diazo dye, which becomes de-coloured in contact with any antioxidant chemicals (Crawford 1998). A positive result for antioxidant chemical presence was thus a clearing reaction from pink to clear in regions of antioxidant activity on membrane prints. The method was to dissolve 0.5 g of DPPH (Sigma) powder in 250 ml of absolute ethanol and pour into a hand-pumped aerosol sprayer. After hand pressurising the sprayer, a fine mist of DPPH solution was sprayed evenly over tissue prints within a cardboard box within a fume cupboard and a photograph taken after 30 seconds.

4.3.6.1.2 Peroxidase assay tissue print method

Fungal peroxidases are an important group of enzymes involved in catabolic reactions that involve H_2O_2 and the production of free radical intermediates. Peroxidases are active outside hyphae (Kuerk 1991, Rothschild *et al.* 1998) and several families of lignin peroxidases catalase the breakdown of this woody macromolecule

(Ward 1998 & chapter 1). The basis for the peroxidase detection assay, one of the enzymes involved in the catabolic breakdown of lignin (Ward 1998, Rothschild *et al.*1998), was a detection solution containing aqueous guiacol and hydrogen peroxide. The colour of guiacol is normally clear. However, on breakdown of hydrogen peroxide by peroxidase, reactive oxygen intermediates (ROI) are produced that transform guiacol into a brown-blue coloured product (Grumass, Gouther & Fritsch 1998, Heinfling *et al.*1998). The detection solution was 0.1 % H_2O_2 in 10 mM aqueous guiacol solution made in 50 mM phosphate buffer (pH 5.3) prepared freshly for each assay by adding 25µl guiacol (Sigma chemicals) to 67µl 30 % H_2O_2 (Sigma chemicals) made up to 20 ml in phosphate buffer which was prepared as follows. Citrate phosphate buffer (50 mM with respect to Phosphate) was prepared by dissolving 2.84 g of Na_2HPO_4 in 100 ml milliQ water to make 0.2 M stock from which 12.5 ml was further diluted to 50 mM by making up to 50 ml in milliQ water. With the aid of a digital pH probe, the final pH of the phosphate buffer was adjusted to 5.3 using drops of citric acid. Buffer and detection solutions were kept in labelled measuring cylinders. A developing trough was made ready with Whatman filter paper in the bottom soaked in buffer. The method used to develop tissue prints was to take a print, place it on a buffer-soaked piece of filter paper, and spread 1.5 mls of detection solution over it. The reaction was allowed to proceed for 5 minutes before photographing any guiacol colour changes, indicating regions of fungal peroxidase activity.

4.3.6.1.3 Free radical assay method

NBT tissue print: The basis of this free radical assay (adapted from Sollod, Jenns & Daub 1992) was the use of a solution of the tetrazolium dye p-Nitro Blue Tetrazolium (NBT) which turns from colourless to a deep blue colour on interaction with any free radical ROI. Free radicals scavenge the dye's electrons (the dye is thus reduced) and this causes colour change by altering the frequencies of light absorbance of NBT's chemical structure. 0.025 g of NBT were dissolved in 25 ml milliQ water. This was poured into a hand-pumped manually pressured aerosol sprayer, pressurised, and sprayed evenly in a fine mist over the tissue print in cardboard box within a fume cupboard. A photograph was taken after 3 minutes.

4.3.6.1.4 Catalase assay tissue print method

Catalase is an important enzyme inside hyphal protoplasm that cleaves H_2O_2 into water and oxygen and is located within organelles called peroxisomes and glyoxisomes (Kunau 1998). The basis of this tissue print assay is that catalase breaks down H_2O_2 into O_2 and H_2O and thus prevents H_2O_2 from reacting with thiosulphate. This leaves thiosulphate in a state whereby it can covert iodine to iodide. Iodide, unlike iodine, cannot react with starch to form a blue chromatophore. On the other hand, without catalase, H_2O_2 acts on thiosulphate rendering it unreactive with iodine. The unaffected iodine develops a blue chromatophore in areas with no catalase activity. Tissue print membranes had to be specially pre-prepared for this assay. Starch membranes were prepared by impregnating 9 cm Petri dish diameter nitrocellulose membranes by evenly mixing in a 1 % starch solution (made by adding 10g soluble starch in 100 ml milliQ water and heating in microwave) for one minute. The membranes were then lifted out of starch solution with forceps and blotted dry on filter paper. After completely drying, the membranes were ready for printing. Printing occurred for 3 - 4 minutes in the way described above.

Three developing solutions were prepared, 60 mM concentration of thiosulphate solution A, 3 % concentration of H_2O_2 solution B, and 90 mM concentration potassium iodide in 0.5 % glacial acetic acid as

solution C. Solution A was made by dissolving 2.98 g of bench stock sodium thiosulphate powder in 200 ml milliQ water in a measuring cylinder. To make Solution B, 3.5 ml 30 % H_2O_2 was made up to 35 ml with milliQ water in a measuring cylinder. Solution C was made by taking 7.47 g bench stock potassium iodide powder and dissolving it in 450 ml milliQ water in a measuring cylinder. 2.5 ml acetic acid was added to the measuring cylinder and the whole solution was then brought up to 500 ml with milliQ water.

The method used to develop tissue prints was to freshly mix 15 ml of solution A and 35 ml of solution B in a labelled developing trough. A fresh tissue print using the starch-impregnated membrane was completely immersed in this solution. After two minutes the membrane was removed with forceps and transferred to 50 ml of solution C in another developing trough. The membrane was swirled and turned whilst keeping it immersed. Initial blue stain begins to fade developing clear zones of catalase presence after 3 minutes. Positions of oxygen bubbles were also noted. The print was then dabbed on clean towelling and photographed after 12 minutes. Prints become more intense after 10 minutes but fade after 30 minutes.

4.3.6.1.5 H_2O_2 localisation assay method

The basis for this H_2O_2 localisation tissue print (adapted from Schopfer 1994) is the H_20_2 mediated reaction which changes colourless iodide to iodine - which then turns blue in the presence of starch. This test is essentially a mirror of the catalase tissue print assay (above), but instead of clear areas in a sea of blue, the positive test for H_2O_2 is an iodine blue-brown zone that occurs in a sea of white pre-impregnated starch iodide paper. Consequently there was no additional developing solution required in this assay since the membranes were themselves pre-impregnated with the required reactives. Pre-impregnated nitro-cellulose papers were prepared no longer than 2 hours before tissue printing by immersing 9 cm Petri dish diameter membranes in a solution of 1 % starch and 0.5 M potassium iodide in a large plastic beaker. To achieve this, 10g bench stock soluble starch was added to 100 mls milliQ water in the beaker and heated until in solution. Whist cooling, 8.3 g bench stock KI powder was added and stirred with a glass rod until dissolved. The 9 cm Petri dish diameter nitro cellulose membranes were immersed in this solution for 5 minutes whilst still warm, mixed under immersion, removed and dried in a dark, clean cardboard box with paper towelling inside a fume cupboard. Membranes were then wrapped in aluminium foil to keep out light. Auto-oxidation of membranes from iodide to iodine occurs with UV frequencies of natural light, so it was important to keep the membranes in the dark until printing. Printing occurred for 4 to 6 minutes (as described above). All prepared membranes were used within 2 hours of preparation. After printing, membranes were placed on clean filter paper and photographed after 2 minutes.

4.3.6.2 HPLC (high pressure liquid chromatography) methods

Five 5 mm diameter plugs were removed from fungal cultures in experiments with and without cecids and at different ages and states of pigmentation. One plug was taken 4 cm from the edge of the Petri dish from each of 5 repeat cultures. Plugs were aseptically removed with a stainless steel hole borer and blue Bunsen flame. The plugs were transferred aseptically using a tungsten filament with handle to small, labelled screw cap

glass cuvettes with 3 cm^3 100 % HPLC grade ethyl acetate. Cuvettes were shaken overnight at 25 °C in a lightproof box covered in aluminium foil. The content of cuvettes were carefully numbered so as to keep track of sample identities right through the process until injection into the HPLC device. Firstly the samples were evaporated to dryness using a freeze-dryer (Crowe 1997) by transferring 7 ml of original ethyl acetate

extracts into 9 ml disposable glass tubes and loading into the freeze drier rotor to be spun under vacuum until dry. On removal from the freeze drier, dried samples were re-suspended in 820µl 100 % HPLC -grade methanol (Rathburn Chemicals Ltd) and adjusted to a ratio of 117 µl 100 % HPLC grade methanol per 1 ml ethyl acetate in the original samples (Watkins 1998). The base of each concentrated sample was briefly immersed in an ultrasonic bath to re-suspend contents more quickly. Each sample was effectively concentrated 8.6 fold to give a suitable volume for qualitative and quantitative HPLC analysis. 200µl of resuspended sample was then syringe filtered to remove particulate matter through sterile 0.22 nylon (Whatman) disposable filters into PTFE-capped HPLC vials. These were labelled with correct sample identities and loaded sequentially into an automated HLPC vial holder. Samples were automatically loaded with 100 µl injection loop volume onto a 250 mm x 4.6 mm Spherisorb C18 reverse phase HPLC column (Anachem ODS2) with a Gilson Automated Sample Injector, and eluted using one of two solvent gradients 1 and 2.

HPLC solvent gradient 1 ran in two slopes from 100 % solvent A (solvent A consisted of 2 litres of a freshly prepared milliQ water based pH buffer consisting of 0.1 M $NH_4H_2PO_4$ + 3 ml concentrated H_3PO_4) to 100 % solvent B (solvent B consisted of 1 litre 75 % HPLC grade acetonitrile Far UV - Rathburn Chemicals Ltd). The first slope took the gradient from 100 % solvent A to 75 % solvent B in 19 minutes, followed by increasing speed of solvent B's introduction remixing to 100 % solvent B by 21 minutes. There followed a 10-minute wash in 100 % solvent B before a steep remixing to achieve 100 % solvent A from 29 to 31 minutes. The advantage of this solvent system was that eluted UV absorbate could be quantified at 2 wavelengths: 210 nm and 280 nm on HPLC detector 1, or 3 wavelengths 215 nm, 280 nm and 350 nm with detector 2 (both machines having been calibrated to this particular gradient system). Also, when using detector 2, an optional entire UV light wavelength absorbance profile could be produced across all UV wavelengths for any particular chosen peak highlighted in the current chemical profile. Flow rate was adjusted to 1 ml min^{-1}. The eluate from the HPLC column passed through a UV Gilson UV116 detector. Data were collected using Gilson 712 HPLC controller software Version 1.20 (Gilson Medical Electronics Inc) for Windows 3.1 through an RS232 serial interface connected to an IBM compatible 386 SX Viglen computer with 40 Mb hard drive and 4 Mb RAM.

HPLC solvent gradient 2 (Watkins 1998, Crowe 1997) ran in a straight slope from 100 % solvent A (solvent A consisted of a milliQ water - based pH buffer consisting of 0.1 M $NH_4H_2PO_4$ + 3 ml concentrated H_3PO_4) to 100 % solvent B (solvent B consisted of 75 % HPLC grade acetonitrile Far UV - Rathburn Chemicals ltd) over 20 minutes, followed by a 10 minute wash in 100 % solvent B until 27 minutes, and finally rapid remixing to 100 % solvent A by 29 minutes. Flow rate was adjusted to 1 ml min^{-1}. The eluate from the column was then quantified by UV Gilson UV116 detector. Data were collected using Gilson 712 HPLC controller software Version 1.20 (Gilson Medical Electronics Inc) for Windows 3.1 through an RS232 serial interface connected to an IBM compatable 386 SX Viglen computer with 40 Mb hard drive and 4 Mb RAM.

4.3.7 Statistical Analyses methods

The t-test was used to compare two normally distributed data sets for significant difference ($P<0.05$) of less than 5 % of the difference in variation due to chance. For small sample sizes of less than nine, where normal distribution could not be ascertained, the Mann-Whitney test, a non-parametric t-test equivalent was used.

Regression analyses were made when reactions were dependent on time on the x-axis. MANOVA was used to compare paedogenic Gt and Gno between 4 treatments. Statistical analyses were carried out using Minitab for Windows software.

4.4 Results

4.4.1 Repli-plate experiment results

Figure 4.6 shows the results of a one month repli-plate multiple fungal host choice experiment in which *Brittenia fraxinicola* cecid populations were noted temporally (2 dates) in terms of number of repli-plate wells colonised for each fungal species. The only significant differences overall were that *Hypoxylon fuscum* achieved a significantly higher degree of *B. fraxinicola* colonisation compared to all the other fungi ($P<0.05$). Significant temporal changes ($P<0.05$) were only found for the fungal species *Hypoxylon fuscum* and *Phallus impudicus*, which both showed significant growth in numbers of wells colonised by the latter date. A slight complication was that *Phallus impudicus* and *Phanaerochaete velutina*, unlike the other fungi, were not restricted by the repli-plate partitions and sent rhizomorphs exploring over the repli-plate well walls which interacted with neighbouring fungal species. This means that some wells contained more than one fungal species by the end of the experiment (although most remained with a single species). If cecids were found in such interactive wells, data were scored equally for each fungal species present, which means that there is overlap in the results presented with respect to *Phallus impudicus* and *Phanaerochaete velutina*

4.4.2 Foraging trajectories - results

Figure 4.7 shows trajectories left by *Brittenia fraxinicola* larvae as they traverse aerial mycelium of *Hypoxylon fuscum* and mark out pathways through pigmented agar at the base of 9 cm diameter Petri dish plastic. The shape of emergent foraging patterns made by trajectories of cecids can be seen to attune to positions of thickened and insulated hyphal fusions in figure 4.8 between two somatically compatible, genetically identical isolates of *Celindrobasidion evolvens* (also known as *Corticium evolvens)* which had been cultured from the study site (see previous chapter 3). The trajectories left by 3 cecid species: *Mycophila speyeri, Heteropeza pygmaea* and *Brittenia fraxinicola*, are shown to differ on the same fungal species – *C. evolvens*. Figure 4.9 (4) shows a graph of difference between curvature measurements (ratio of direct path length in mm / actual path length in mm) of *Brittenia fraxinicola* trajectories on single cultures of *Hypoxylon fuscum* and *Vuilleminia commedens*. The graph of curvature (4) with standard errors of means as error bars, shows curvature variance to be non-overlapping between fungal treatments. When subjected to a parametric t-test, the treatments show a highly significant difference ($P< 0.001$) - with less than 1 % of the differences between variances attributable to chance. Images (1) and (3) reinforce this evidence since, at **magnification of x 20 and x 30**, the *H. fuscum* trajectories are just as straight as the *V. commedens* trajectories are at **x 10 magnification.**

4.4.3 Paedogenic life-cycle results

Results of experiments to show effects of changes in fungal environment on the life-cycle of cecids can be seen in figure 4.10. Cecid larvae show a significantly different distribution ($P<0.001$) of both progeny number (Gno) and generation time (Gt) with respect to the four fungal species environments (Tables 4.1 & 4.2). Two fungal environment treatments, *V. commedens* and *H. fuscum*, result in significantly lower Gno.

These same two fungal treatments result in significantly longer Gt ($P<0.001$), with respect to the other two fungal treatments, *Chrondrostereum purpureum* and *Corticium evolvens*. The significance of this grouping of fungal environments was calculated via MANOVA (multiple analysis of variance of mean). The paired *V. commedens* and *H. fuscum* treatments were not significantly different, neither were *C. purpureum* and *C. evolvens* significantly different in their mean Gno and Gt, as a group. However the between pair variation of the group *V. commedens* and *H. fuscum* to *C. purpureum* and *C. evolvens* was highly significant ($P<0.001$) for both Gno and Gt. As well as showing that cecid Gt and Gno are changed by fungal environment, these results also demonstrate a possible developmental relationship between Gt and Gno, such that cecids that live significantly longer tend to produce significantly fewer larval progeny at paedogenic birth.

4.4.4 Image analysis of mycelial development results

Changes brought about by *B. fraxinicola* larvae to the shape and degree of pigmentation of *H. fuscum* cultures (figure 4.5) are shown by regression lines of best fit in figure 4.11 to be dependent on time on the x-axis. The overall area of deep green pigmented melanised mycelium increased significantly more rapidly, as shown by t-test between the variances of the two samples ($P<0.05$) with cecid treatments when compared to controls without cecids. The significance of this difference in speed of *H. fuscum* melanisation is mirrored by a t-test confirmed significantly different ($P<0.05$) rate of reduction of unpigmented mycelium between treated and control groups. The raw data for this experiment was obtained by the cutting and weighing acetate technique described in the methods section. The following images and analyses were performed using the computer-aided image analysis technique described in the methods section. Figure 4.12 shows computerised image analysis of top orientations of 6 Petri dishes from one of several experiments showing the effects of *B. fraxinicola* larval treatments on *H. fuscum* melanisation. Figure 4.13 shows effects of cecid treatments on *V. commedens* insulation. Figure 4.14 shows *H. fuscum* computer image analysis results graphically for each of the 2 orientations of Petri dishes both with and without membrane controls. Graphs 1 and 3 are for the top views, 2 and 4 being for the underside. There is an amalgamated data graph for *H. fuscum* in figure 4.15. This amalgamated data showed significant macro-trends for *H. fuscum* for all data from all orientations, with and without membrane. Student's t-tests confirmed the significant differences between treatments. At the end of the experiment the amount of deeply melanised mycelium was significantly higher with cecids ($P<0.0001$), whilst the amount of unmelanised mycelium was significantly less with cecids ($P<0.0001$).

Figure 4.16 (1) shows, on the basis of Mann-Whitney test confirmation, that cecids significantly increased the amount of insulated aerial mycelium ($P<0.05$) but did not significantly decrease ($P>0.05$) the amount of diffuse aerial mycelium. Trends on the reverse side of Petri dishes are shown figure 4.16 (2). However, differences were not significant from the underside views. Cecids dramatically enhanced the amount of data variability and asymmetry with *V. commedens* too. To view outputs from Minitab for Windows t-test and Mann-Whitney test results for this section of results, please turn to Appendix 6.

4.4.5 Metabolic scale results

Having dealt with macro-visible changes brought to fungal mycelial development by cecids, now come results of biochemical assays and HPLC analysis for single fungal cultures treated with and without *B. fraxinicola* larvae.

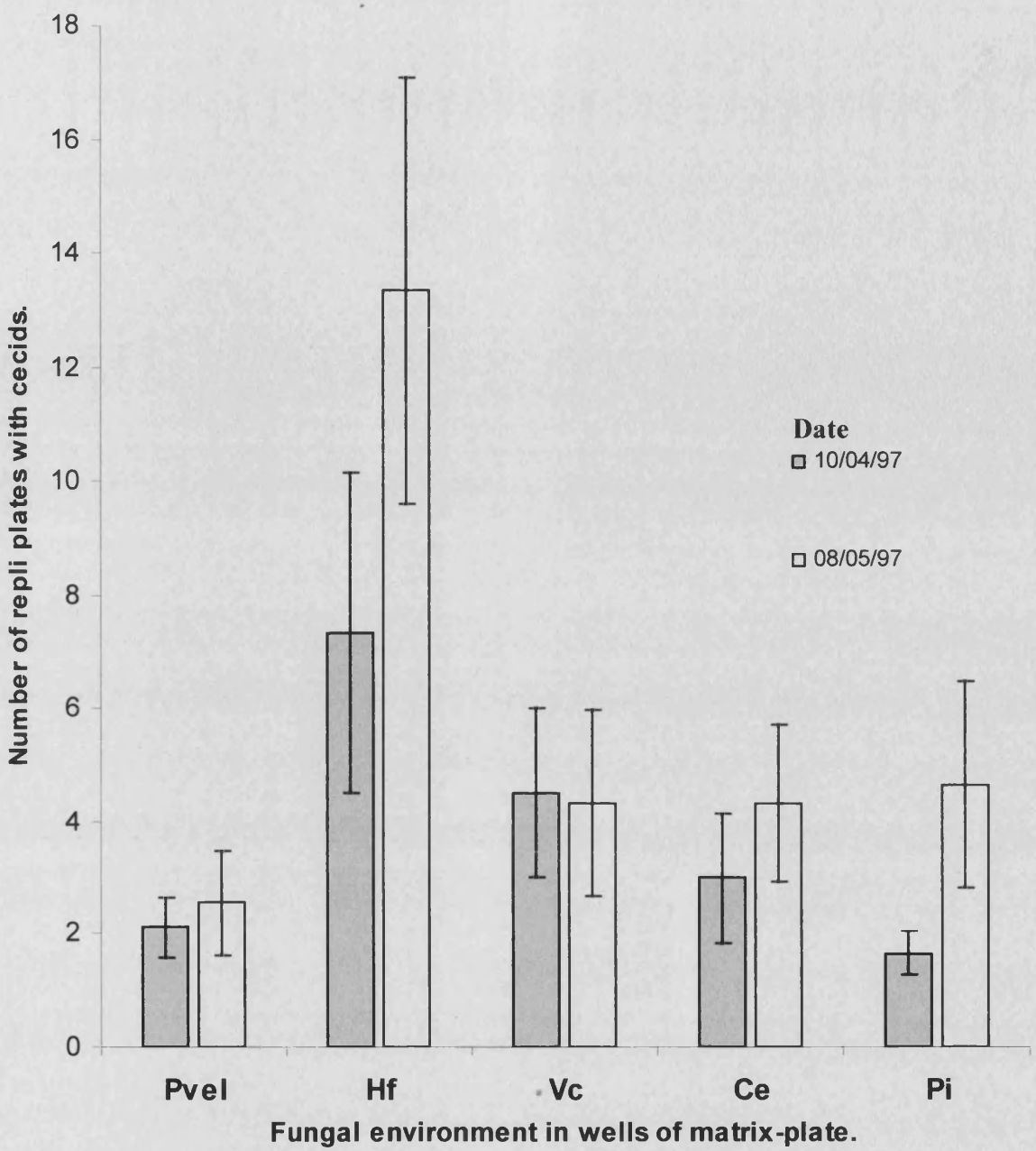
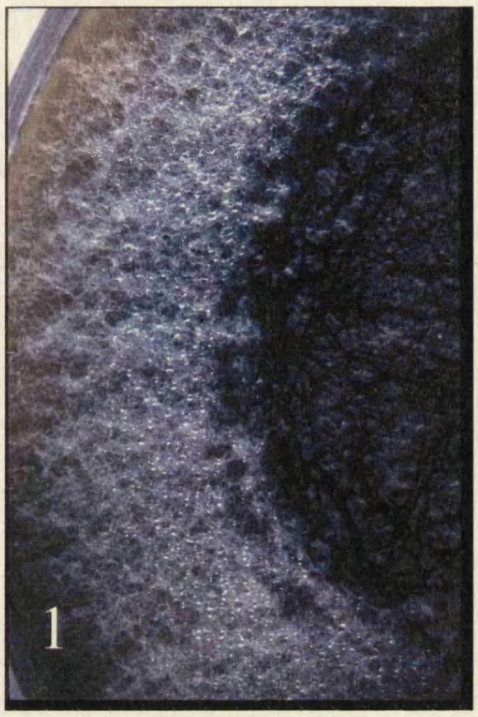


Figure 4.6 Numbers of repli-plate wells with cecids found on two dates for different fungal hosts grown in a repli-plate cecid larval choice bio-assay. Comparison of numbers of matrix plate wells with cecids between fungal environments shows significantly different populations (Mann-Whitney test with n=8) between dates ($P<0.05$) for Hf (*H. fuscum*) and Pi (*P. impudicus*). Also between overall fungal treatments there was a significantly larger population of cecids ($P<0.05$) with *H. fuscum*.



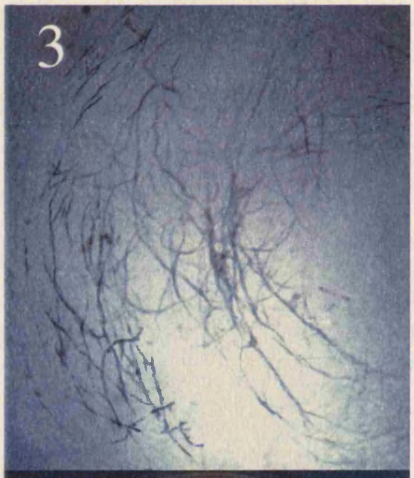


Figure 4.7 Pure cultures of *Hypoxylon fuscum.* 1 shows the radial colony margin reaching the outer edge of the malt agar petri-plate. It is this margin (at left) which is youngest and least pigmented. The older mycelium to the right and centre of the culture is pigmented a deep green colour. It is on this deeper pigmented mycelium that the cecids forage; as can be seen by the Pathways they have created. These trajectories can indicate much about how cecids behave in response to different environments. 2 shows the trajectories on the underside of petri-plates, as does 3; this time the trajectories are remaining after the rest of the agar has been lifted and peeled away. 4 shows progressive *H. fuscum* pigmentation with age from the centre of the petri-dish outwards, as viewed from underneath.

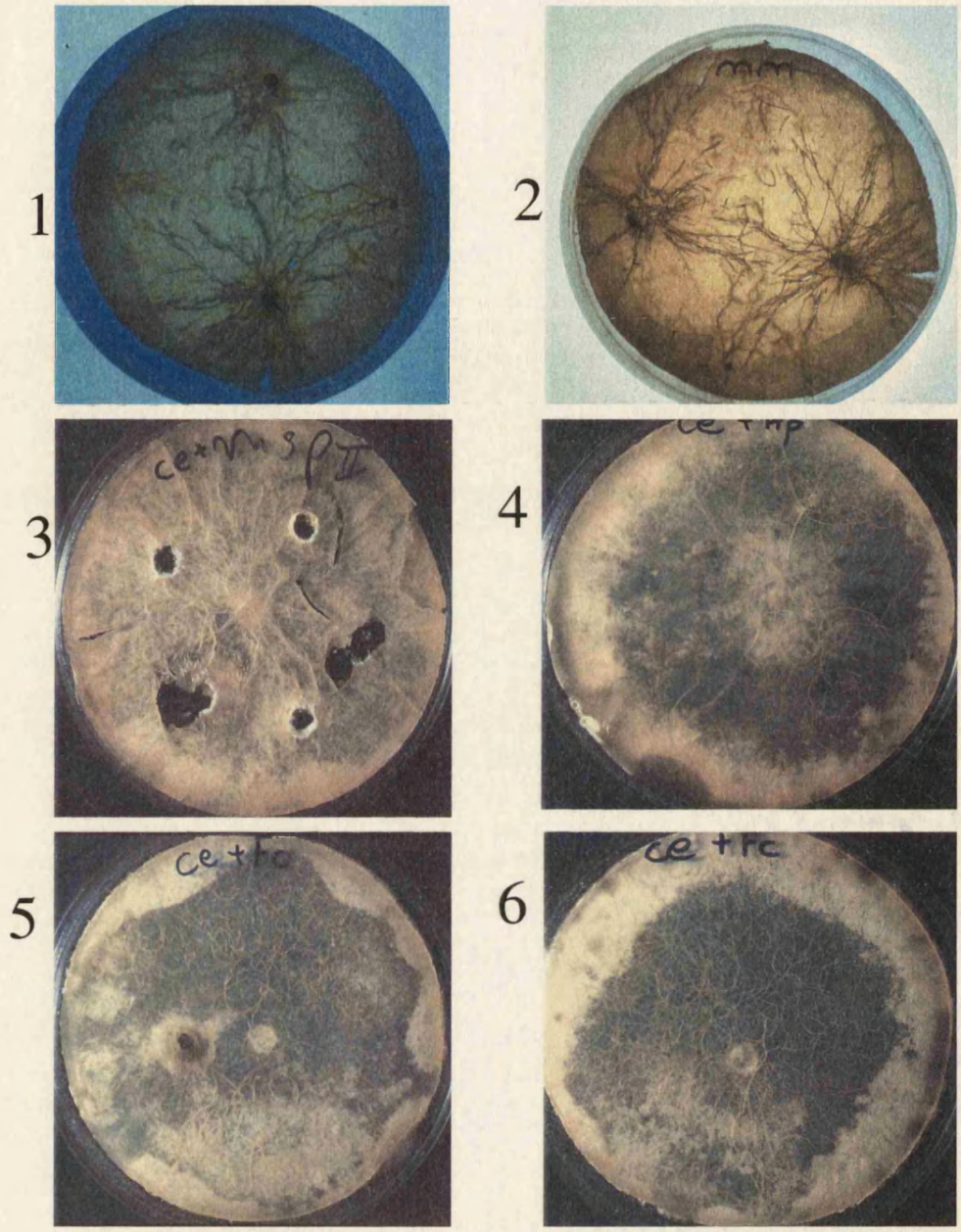
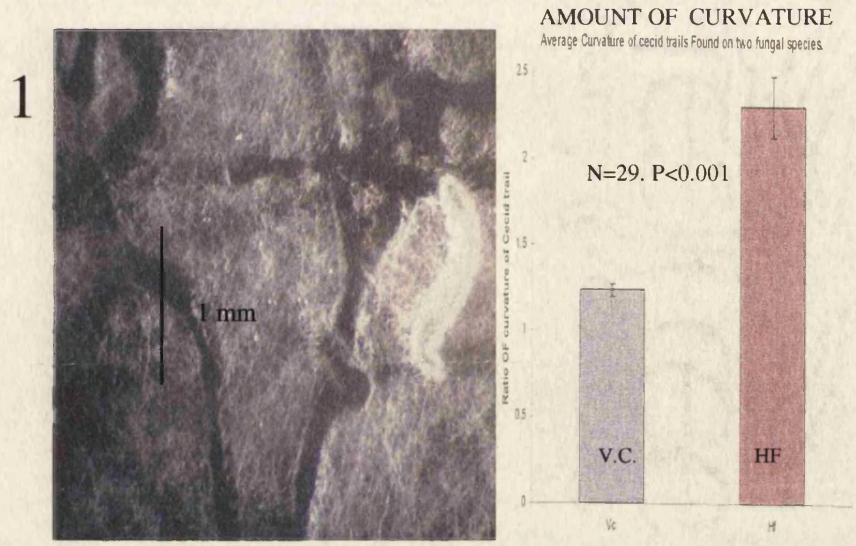
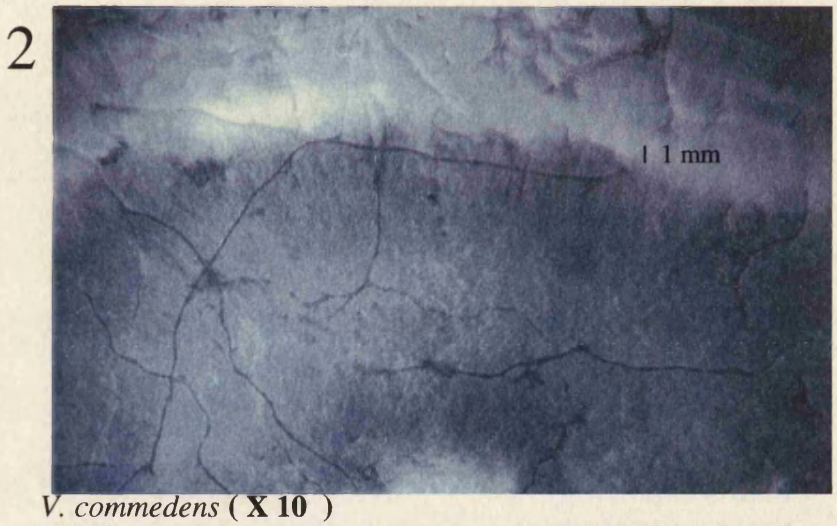


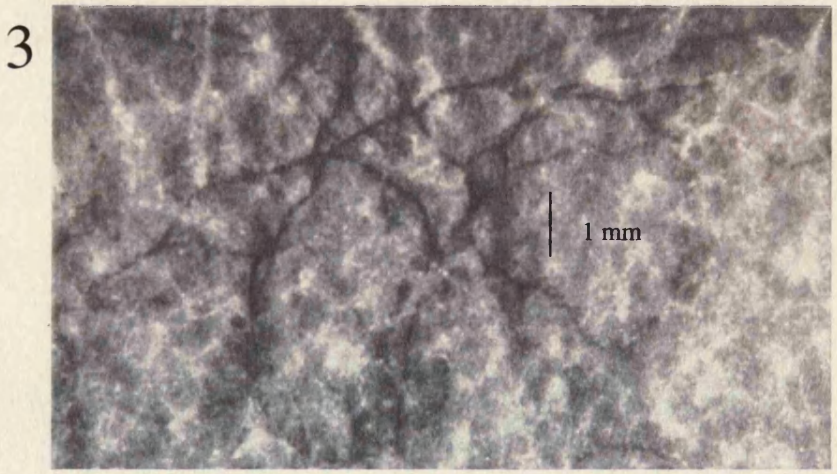
Figure 4.8 Changes in foraging behaviour in relation to the same fungus; *Celindrobasidion evolvens*, isolated from the field; 1+2 show *Mycophila speyeri* foraging trajectories in a bi-inoculation culture of the same genotype. Cecids have created air channels alongside the thickest hyphae; products of hyphal insulation after mycelia have fused. Here the cecid trajectories coincide with those of the fungi. The foraging pattern which emerges from bi-inoculation is markedly different from that in single inoculation (3 - ignore inoculation holes) - where a pattern of foraging loops returns. 4 shows how different cecid species cause different forraging loop patterns; here the loops visibly smaller radii and the cecids are *Heteropaeza pygmea.* 5 and 6 show how the former effect is extenuated with the field cecid *Brittenia fraxinicola.*



Brittennia fraxinicola and it's trajectories over *V. commedens* (**X 30**)



V. commedens (**X 10**)



H. fuscum (**X 20**)

Figure 4.9 (1) shows a *B. fraxinicola* cecid and trajectories made on *V. commedens* (2). (3) Shows trajectories on *H.. fuscum.* Student t-tests of measurements (4) show significant difference P<0.001 in trajectory curvature between fungal environments (n=29).

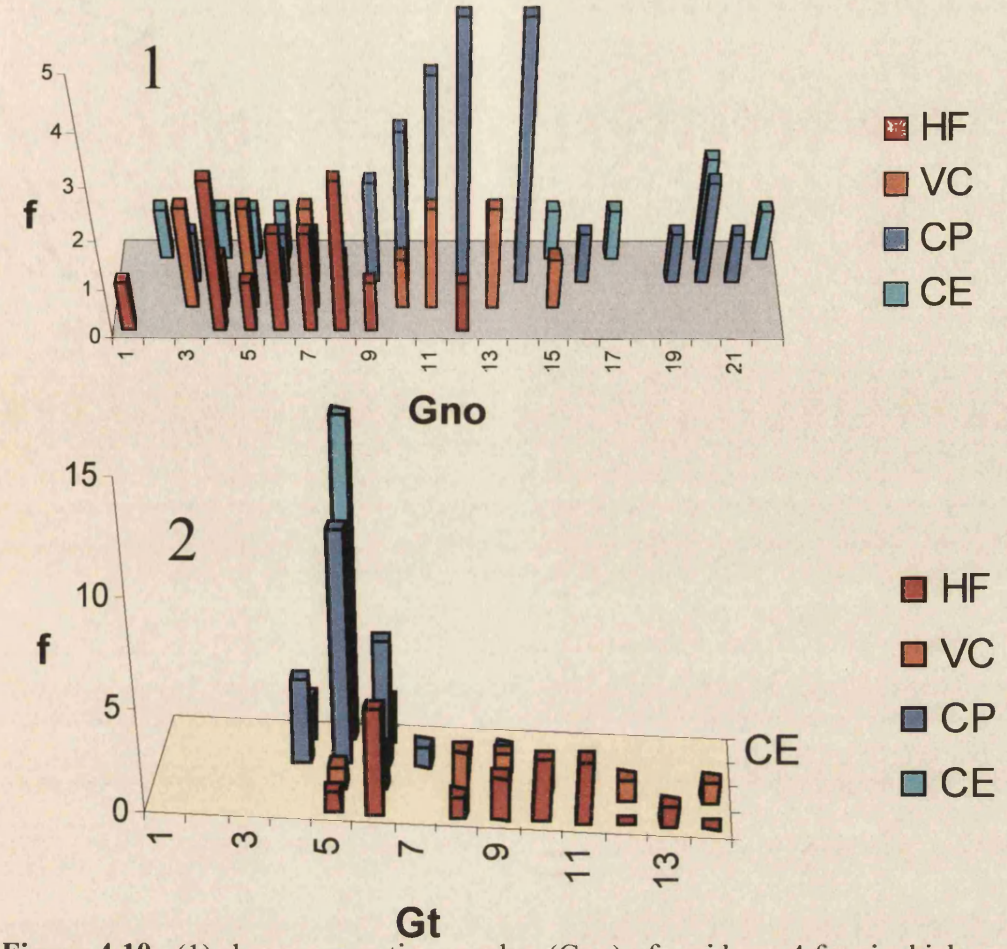


Figure 4.10 (1) shows generation number (Gno) of cecids on 4 fungi which can be grouped into two groups with low Gno (red) and high Gno (blue). (2) shows generation time (Gt) of cecids on 4 fungi which can be grouped into two groups; one with low Gt (blue) and the other with high (Gt). Significance for data set (1) (MANOVA test) is shown in table 1. Significance for data set (2) (MANOVA test) is shown in table 2.

TABLE 1: MANOVA Gno

css/3: general manova		LSD TEST; variable G_NO Probabilities for Post-Hoc Tests MAIN EFFECT: FUNGUS			
FUNGUS	GEN	{1} 7.183333	{2} 6.729167	{3} 12.21667	{4} 13.12500
VC	{1}		.762841	.000303	.000070
HF	{2}	.762841		.000097	.000023
CP	{3}	.000303	.000097		.440473
CE	{4}	.000070	.000023	.440473	

TABLE 2: MANOVA Gt

css/3: general manova		LSD TEST; variable G_T Probabilities for Post-Hoc Tests MAIN EFFECT: FUNGUS			
FUNGUS	GEN	{1} 9.066667	{2} 8.416667	{3} 5.600000	{4} 4.958333
VC	{1}		.372476	.000001	.000000
HF	{2}	.372476		.000039	.000003
CP	{3}	.000001	.000039		.260379
CE	{4}	.000000	.000003	.260379	

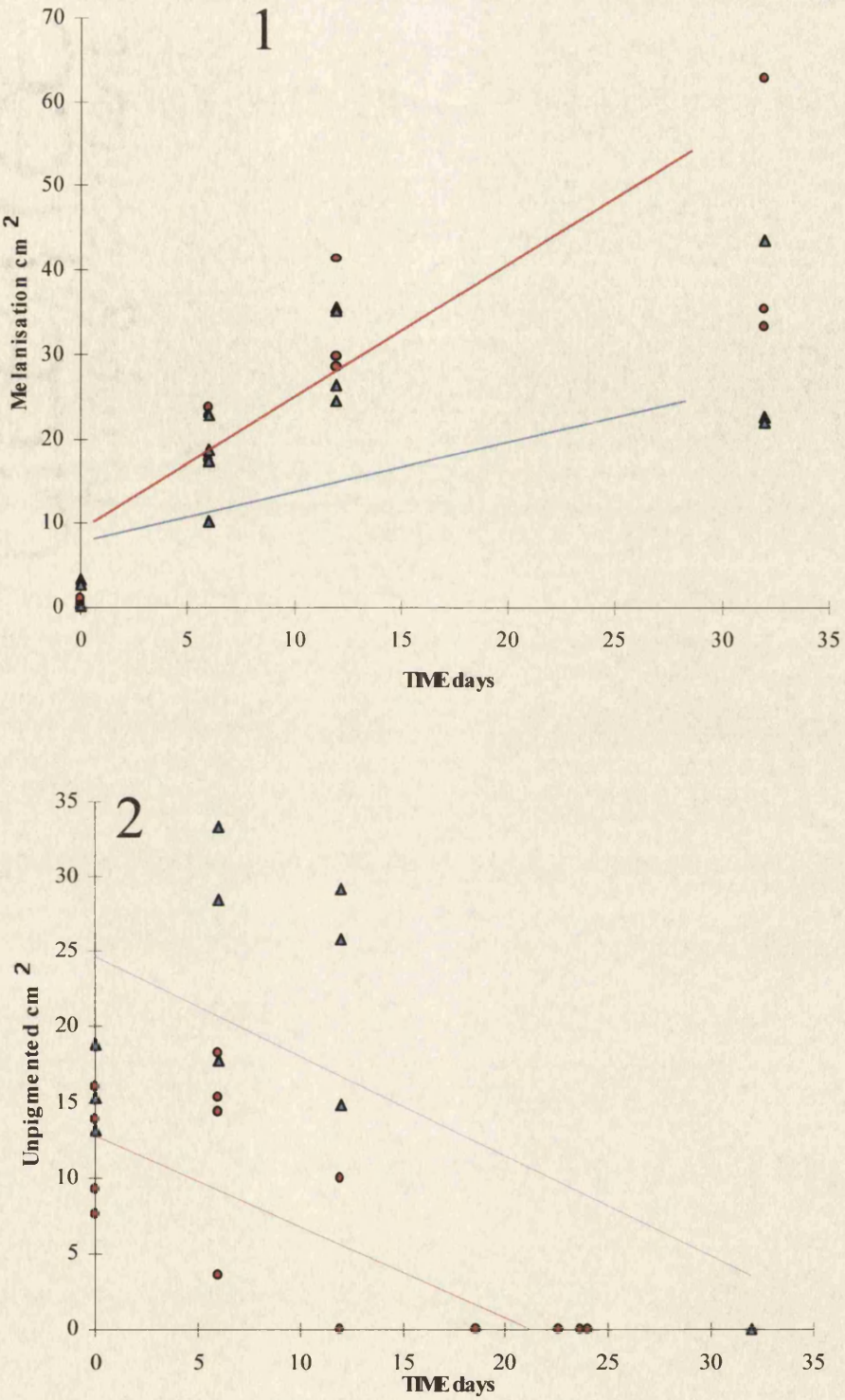


Figure 4.11 Regressions of (1) pigmentation (melanisation) over time b= 1.2 P<0.05and (2) area of mycelium left in an unpigmented state over time, with cecids (red lines), and with controls (blue lines) b= -0.53 P<0.05. The cecid used was *Brittenia fraxinicola* and the fungus *Hypoxylon fuscum.* Regressions were all found to be significant and different in terms of overall data variance, as confirmed by using T-tests (P<0.005).

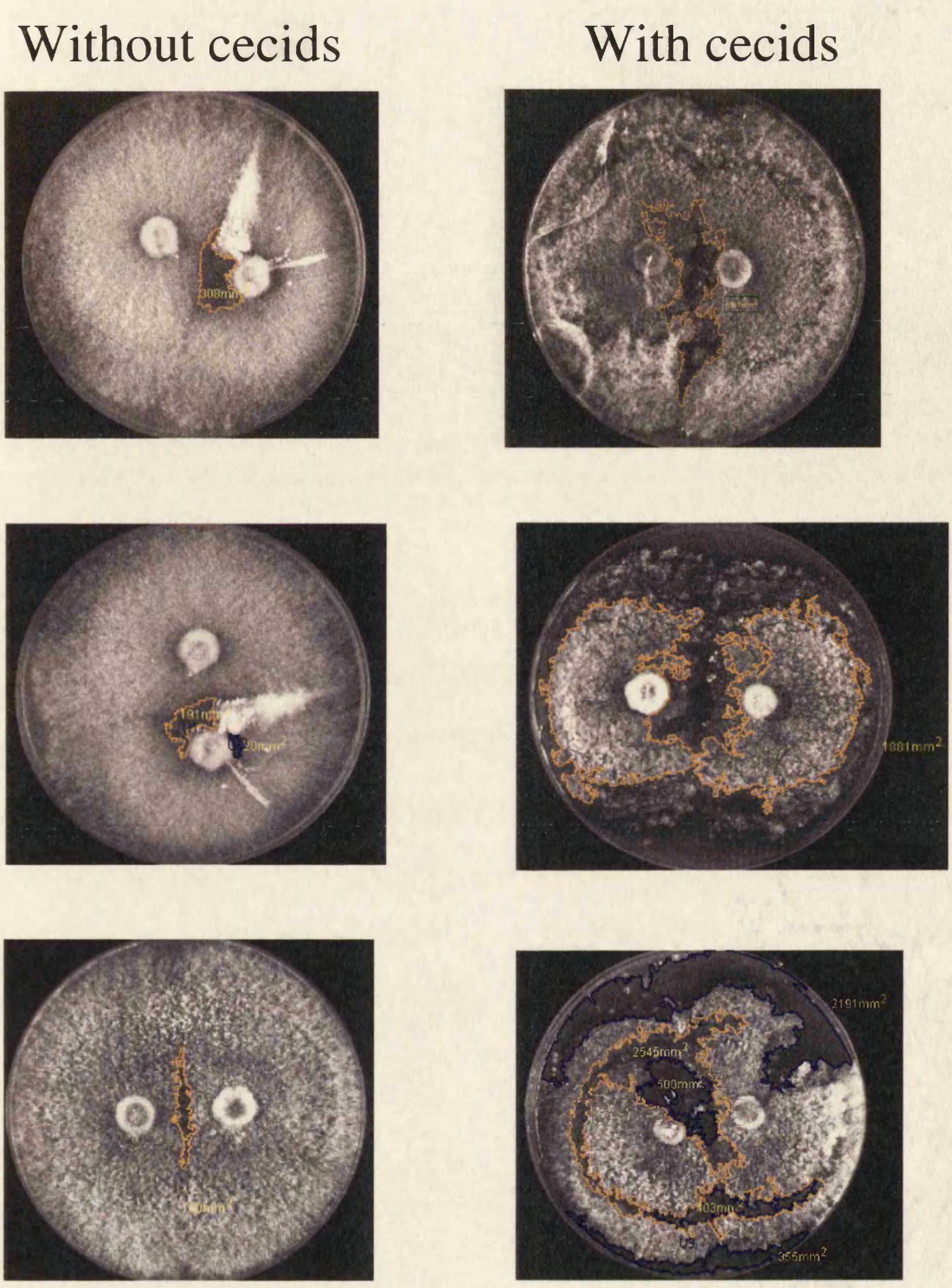


Fig 4.12: Mycelial development contour mapping of pure cultures of *Hypoxylon fuscum* treated with and without cecids.

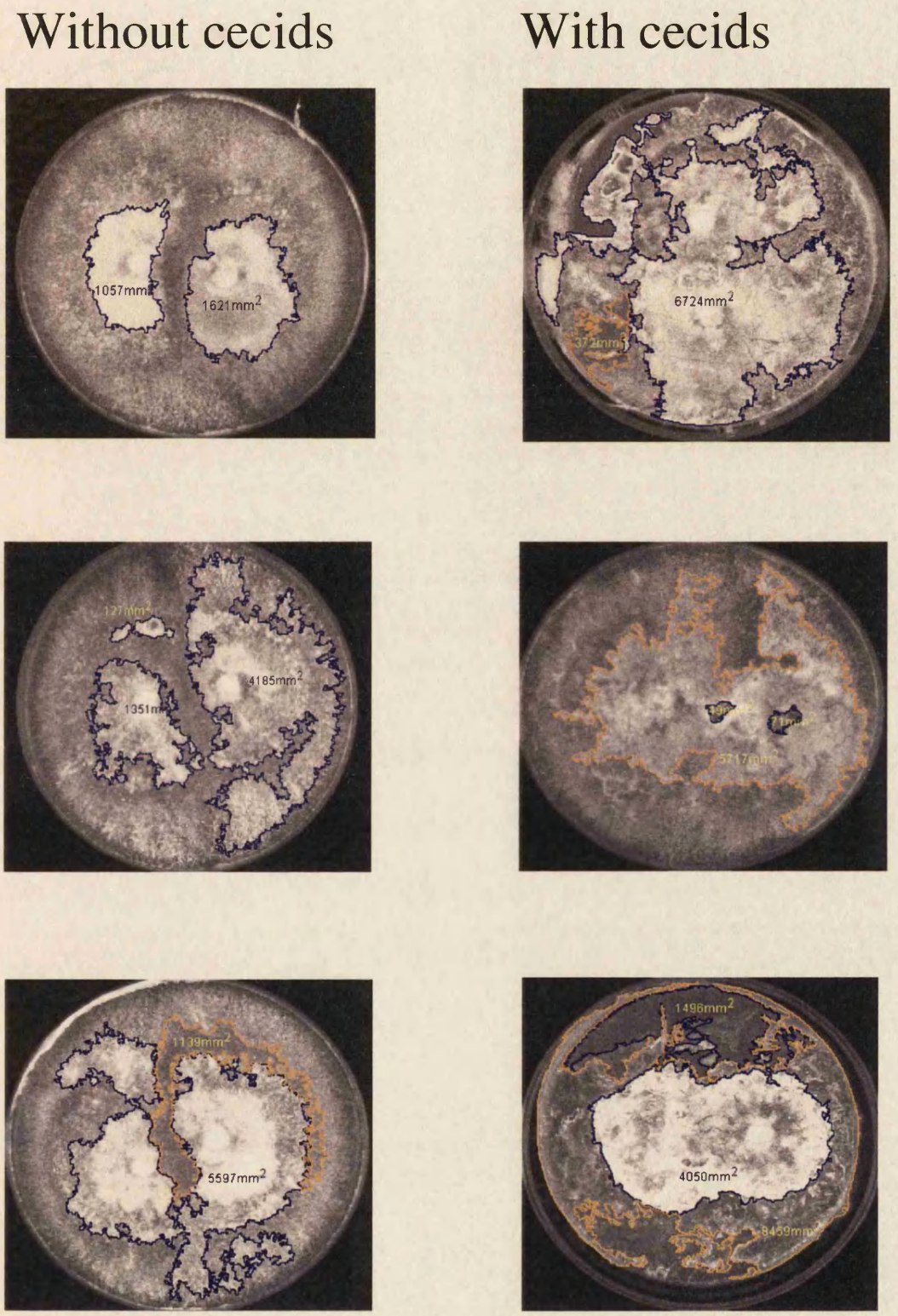


Figure 4.13: Mycelial development contour maps of pure *Vuilleminia commedens* cultures treated with and without cecids.

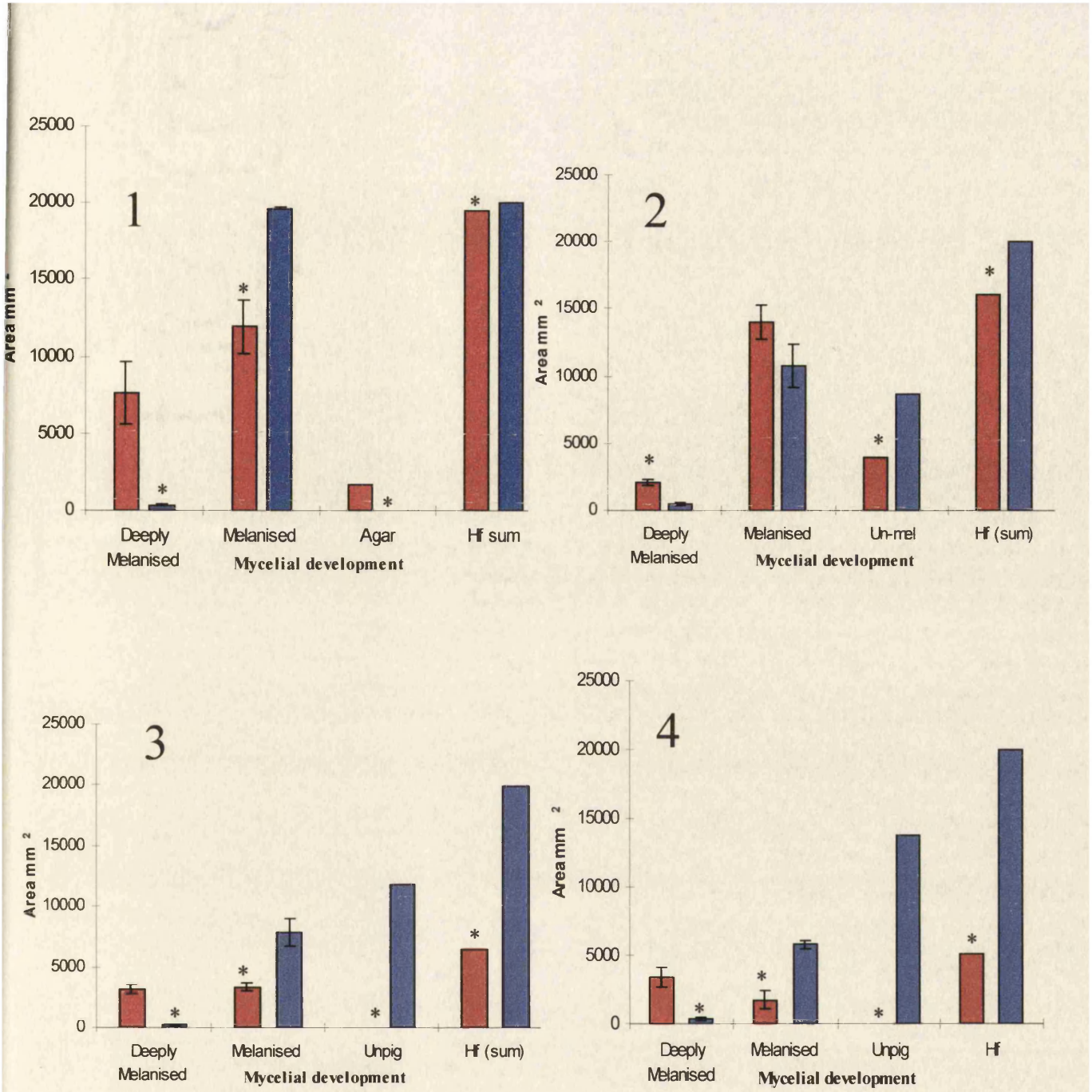


Figure 4.14 (1) shows pigmentation (melanisation) occurring with and without cecids in *H. fuscum* (n=9, P<0.005). (2) shows pigmentation of *H. fuscum* petri-plate undersides (n=8, P<0.05). (3) shows pigmentation occurring (n=9, P<0.001) with tissue print membranes in place. (4) shows membrane plates from the underside (n=9, P<0.005). In each graph the red data sets are for treatments with cecid larvae whist the blue are controls. Standard error bars are shown. Cultures were incubated for one month. With a sample size of 9, Mann-Whitney non-parametric tests showed significance between cecid-treated and controls. Asterisks (*) show significant difference between the two treatments at P<0.05.

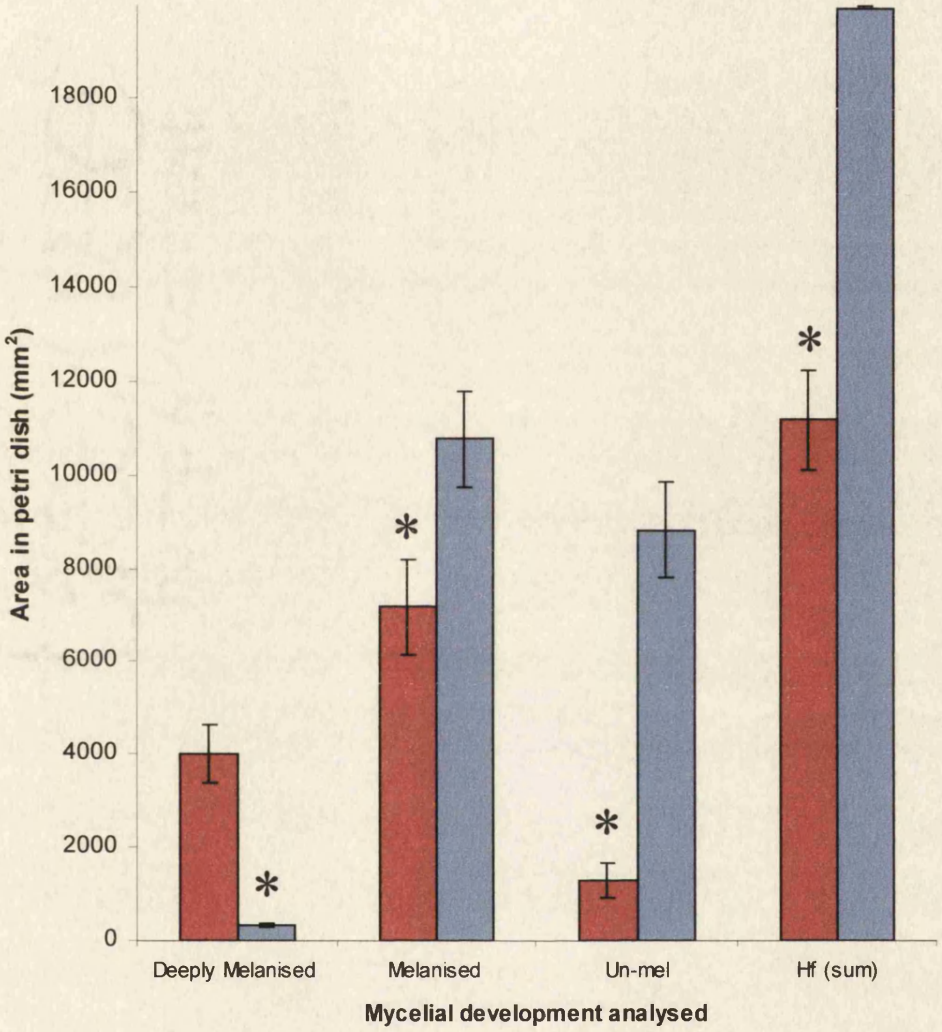
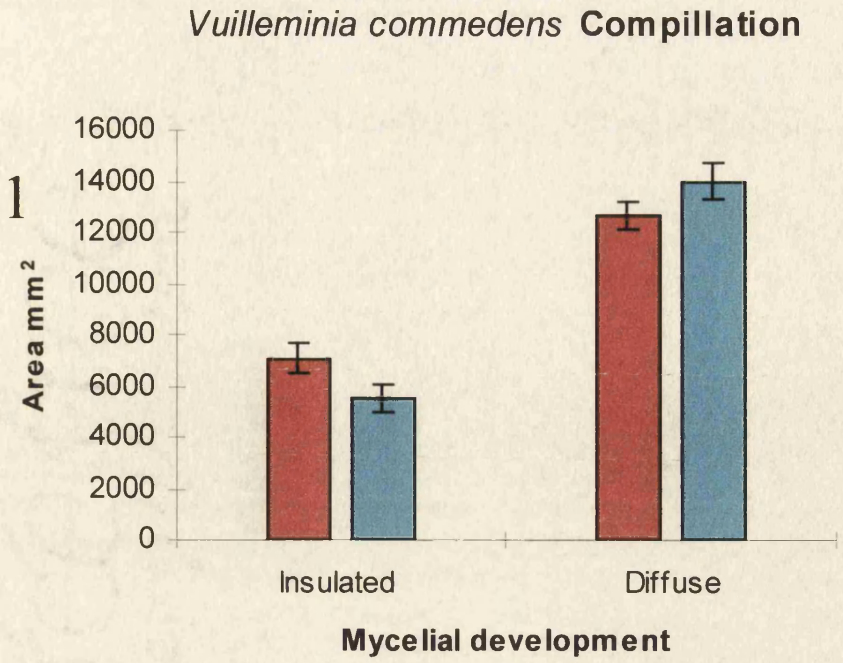


Figure 4.15 Amalgamated trends (n=34) of mycelial development on pure *Hypoxylon fuscum* cultures treated with and without cecids. Red data sets are for cecid-treated cultures whist blue are for controls. Using paired t-tests, significant differences between all ($P<0.05$ asterisked) treatments can be seen with deeply melanised areas ($P<0.0001$), melanised (green) areas ($P<0.001$), unmelanised (un-mel) ($P<0.0001$) and also total culture area (Hf(sum)) $P<0.0001$. Standard error bars are shown.

1



2

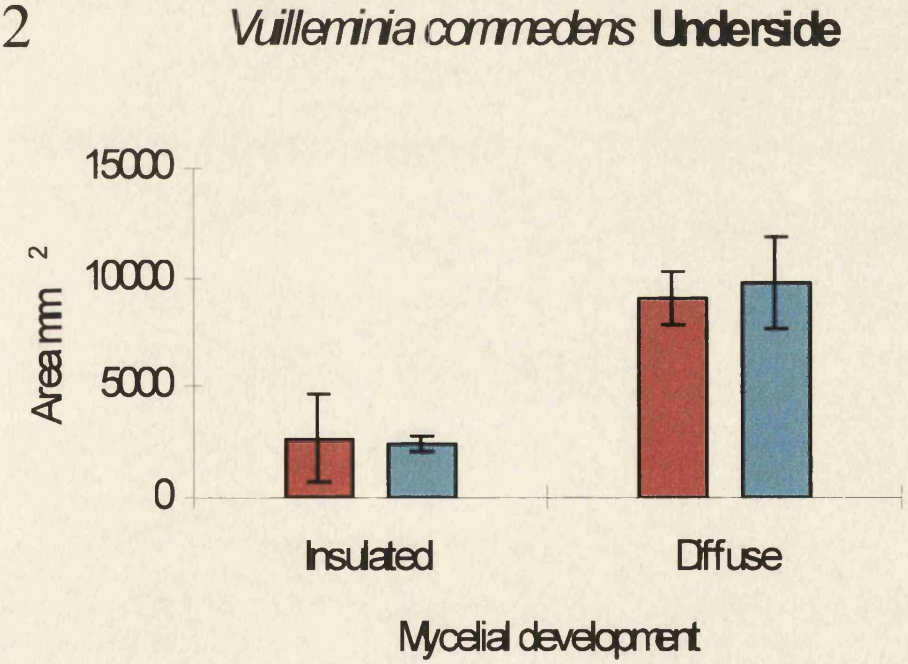


Figure 4.16 Area trends for pure cultures of *Vuilleminia commedens* treated with and without cecids. (1) shows surface view data. (2) shows underside view data. Red data are those treated with cecids, blue are controls. Mann-Whitney tests show significant differences of means between insulated states of fungus at (1) ($P<0.05$ $n=6$), but not (2) ($P>0.05$ $n=3$).

4.4.5.1 Tissue-lysis printing results

Figures 4.17 to 4.20 show photographic results of tissue-lysis print assays, whose main results are summarised in the table 4.3 below. Relative degrees of staining were compared between treatments in order to ascertain whether the cecid-treated cultures increased or decreased relative amounts of metabolic reactants. This was not to quantify but to qualify the direction of response induced by cecid treatments relative to controls.

Table 4.3 Summary of main effects of cecids on the biochemistry of fungal mycelia in single (non interactive) culture

Tissue Printing assay used	Effect of cecids on *H. fuscum*	Effect of cecids on *V. commedens*
DPPH – antioxidant	increased antioxidants relative to controls	increased antioxidants relative to controls
Peroxidase	decreased activity relative to controls	decreased activity relative to controls
Catalase	increased activity relative to controls	increased activity relative to controls
H_2O_2	reduced activity relative to controls	reduced activity relative to controls

The NBT tissue print on *H. fuscum* shown in figure 4.17 (5) shows that free radicals are produced in the most assimilative part of the mycelium where the digestion of nutrients takes place extra-cellularly.

4.4.5.2 High Pressure Liquid Chromatography (HPLC) analysis results

Figures 4.21 to 4.23 display the HPLC metabolite elution profiles for *H. fuscum* and *V. commedens* single cultures as affected by age and the presence of *B. fraxinicola* cecid larvae. Figure 4.21 shows solvent gradient 1 results for *H. fuscum* above those for two ages of *V. commedens*. The only wavelength lines that can be compared between all 3 graphs in shape rather than exact Rf values, are those at 280nm. These are orange lines in the comparison between the two species (1 & 2) and red for the young *V. commedens* culture (3). Old *H. fuscum* cultures (over 2 months since inoculation) produced more non-polar compounds than similarly aged *V. commedens* cultures, which was unexpected. We can also see a large difference in metabolite production between species, with many peaks in common but also many unique to both, which was expected. What is also interesting is to notice an expected shift to the right in non-polar peaks as *V. commedens* ages from young cultures (up to one month since inoculation) to old cultures (more than 4 weeks in age). Figure 4.22 (1) shows differences between solvent gradient systems, species, isolates and treatments of age and *B. fraxinicola* larvae. Top left compares young and old *H. fuscum* isolate 1 (homokaryon) where many expected additional peaks can be seen at the left hand side in the older culture. Shown next to *H. fuscum* isolate 1 is a profile for *H. fuscum* isolate 2 (heterokaryon). As expected for this more deeply pigmented and unstable culture (figure 4.10 & Watkins 1998) more peaks are produced throughout the polarity range, especially at very right (non-polar) and left hand (polar) sides of the profiles which shows that *H. fuscum* complements production of polar molecules with some highly polar ones. At the top right of figure 4.22 (1) are shown *H. fuscum* profiles using solvent gradient 2. A comparison between ages of *H. fuscum* isolate 2 shows a marked increase in polar compounds in the older colony. This change with age is mirrored by the changes brought about by cecids in *H. fuscum* isolate 1. As expected the *H. fuscum* responds

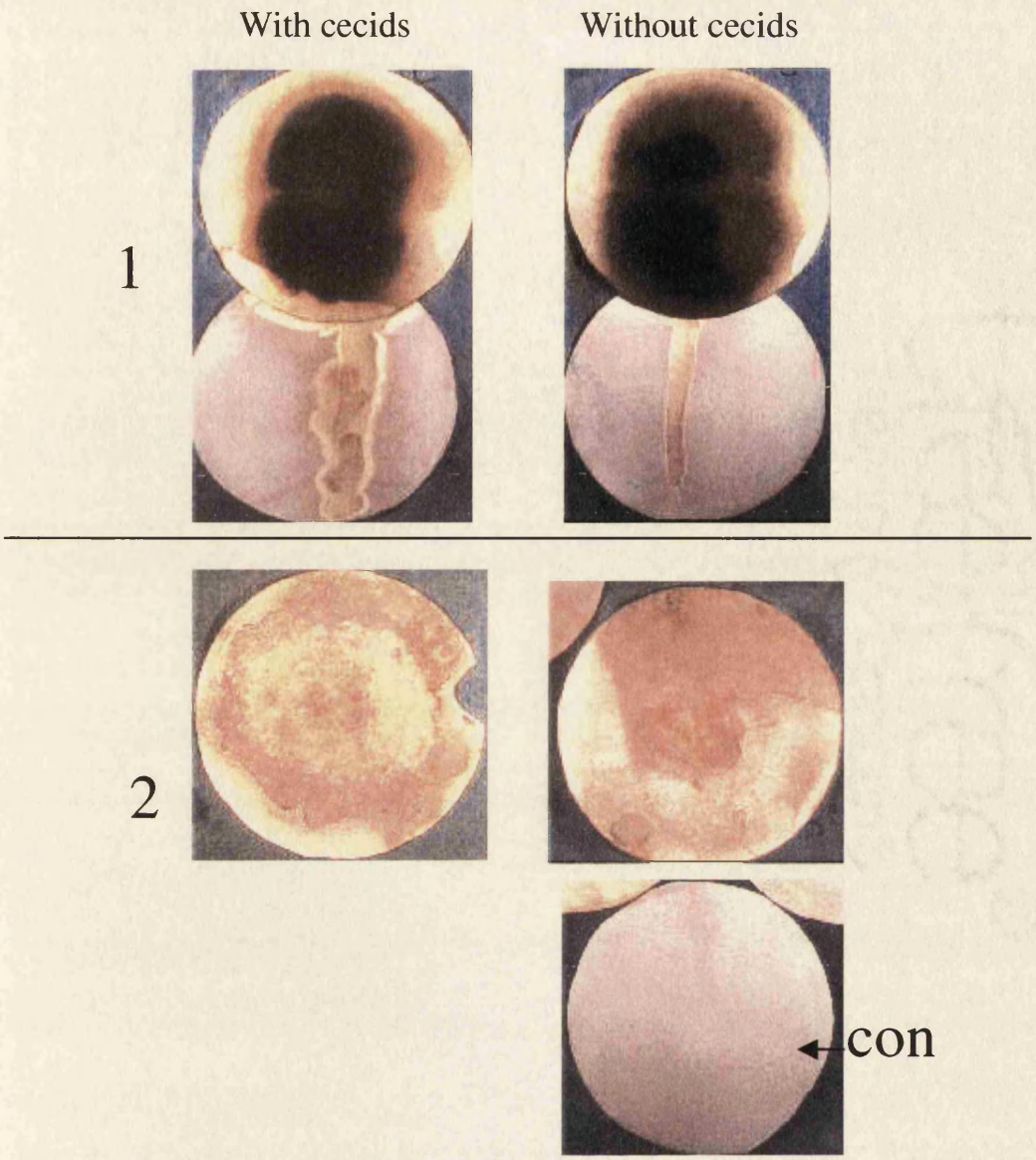


Figure 4.17 (1) shows pure *Hypoxylon fuscum* culture anti-oxidant assay tissue prints from cultures treated with and without cecids. DPPH, the free radical test solution, only remains coloured pink or purple when no anti-oxidants have been encountered. As can be seen above, *H. fuscum* produces copious amounts of anti-oxidants since, compared to controls at the bottom, very little pink remains overall. Some evidence that the green pigments from this fungus have anti-oxidant activity is provided when pigments are made to run over the control membranes, the pink colour is lost immediately and a bright zone of clearing around the pigments then spreads by 2 – 3 mm to either side. This effect is more pronounced with cecids than without.

(2) shows *Vuilleminia commedens* pure culture anti-oxidant tissue prints. The pink free radical DPPH shown on control membrane at bottom right, turns colourless as soon as anti-oxidants neutralise it. It is apparent from the above that anti-oxidants may not be in ready supply in this basidiomycete fungus. If there is any trend it could be that membranes treated with cecids have managed to reduce pink colours better than those treated without cecids; and that the pattern of antioxidant activity in cultures with cecids is also more heterogeneous.

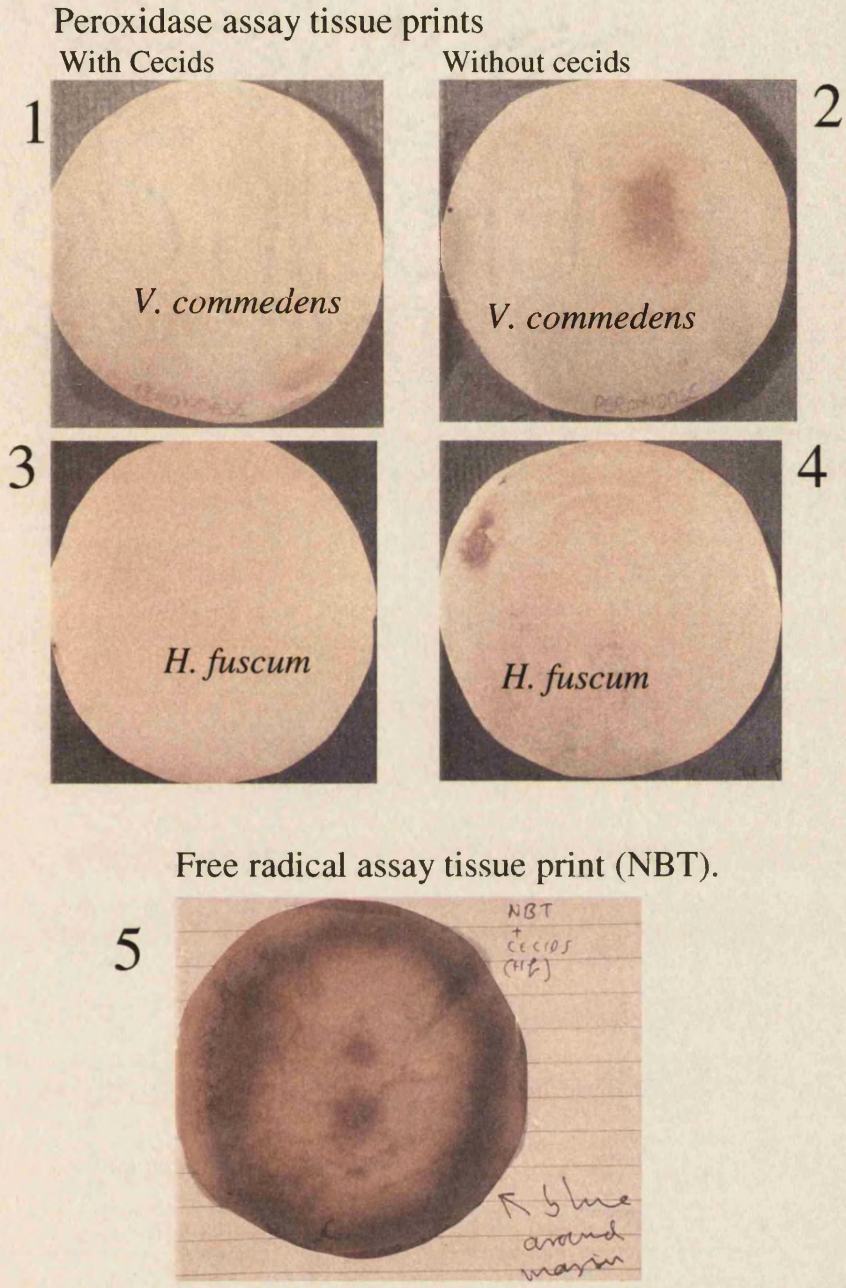


Figure 4.18 Shown at top (1-4) are tissue prints for peroxidase activity in pure cultures with and without cecids. The stain is positive for peroxidase activity with a brown-grey stain. It appears that treatments with cecid larvae repress the activity of peroxidase in these fungi. Below (5) is tissue print to locate free radicals within a mycelium. The stain NBT (an anti-oxidant) is used. A solution of NBT is usually colourless. However, it turns blue on redox reaction with free radicals. It is interesting to see blue around the colony margin of *H. fuscum*.

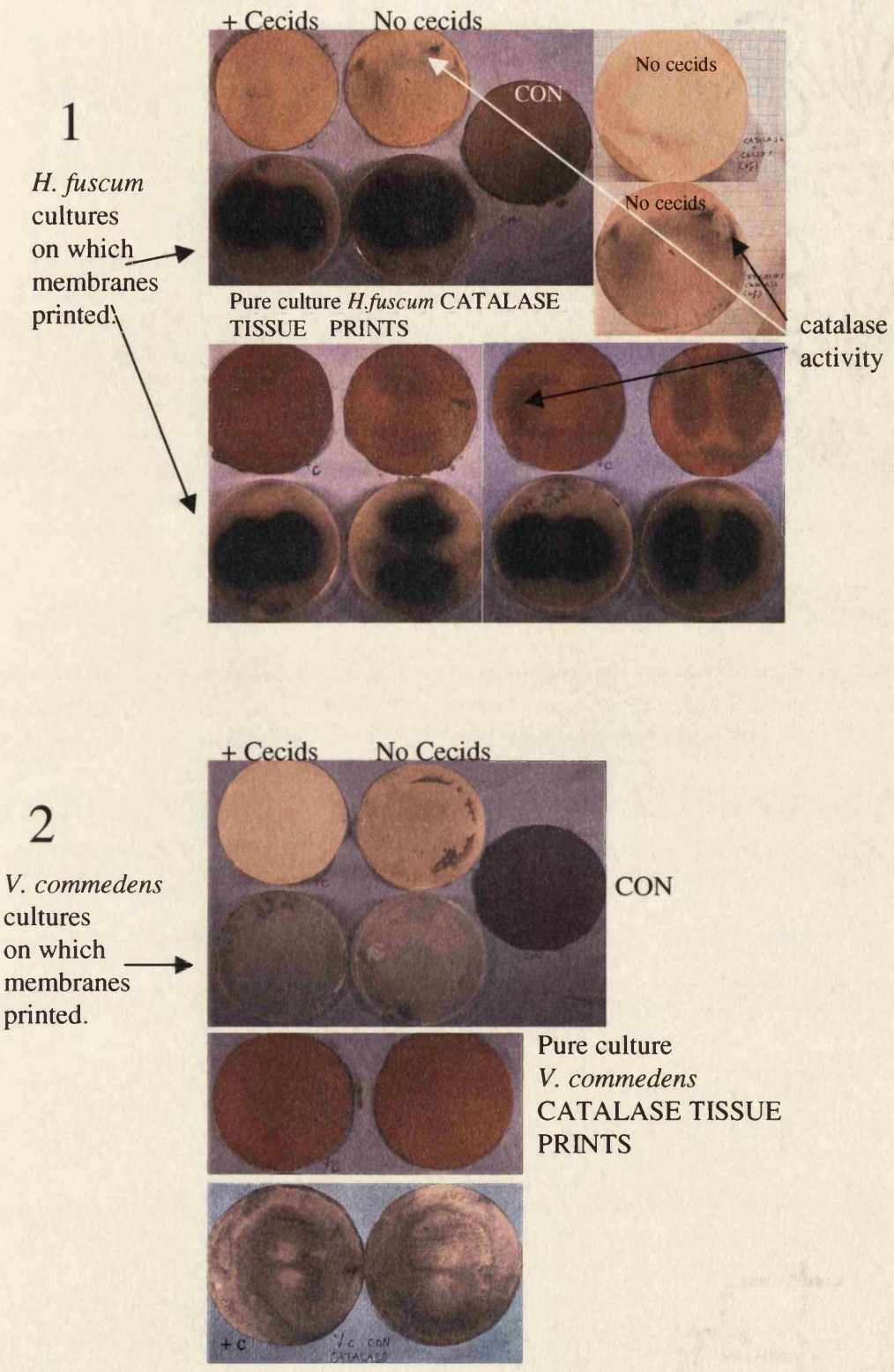


Figure 4.19 Catalase tissue prints showing regions in pure cultures where catalase prevents the action of H_2O_2 on reagents in the test. A positive reaction prevents the staining of white starch-impregnated membrane blue, leaving the locality of enzyme activity white. (1) shows pure cultures of *H. fuscum* with and without cecids, (2) shows *V. commedens* with and without cecids.

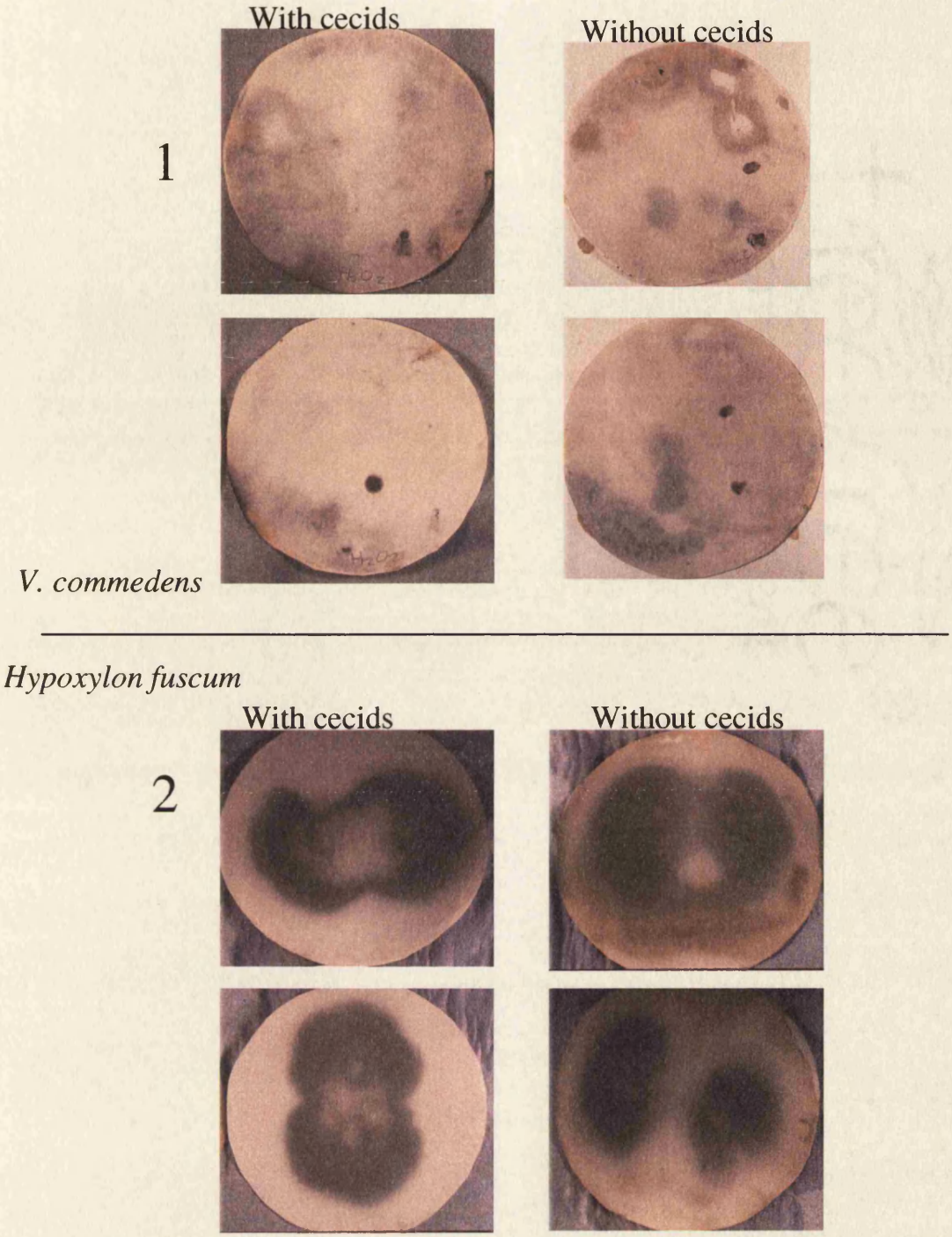


Figure 4.20 H_2O_2 production tissue print assay for pure fungal cultures treated with and without cecids; above the middle line (1) are tissue prints of *V. commedens*, below line; *H. fuscum* (2). Positive test for H_2O_2 creates a brown-orange stain on The regions of the tissue print where H_2O_2 is present. A trend can be seen above Where, for both fungi, treatments with cecids have reduced overall H_2O_2 production. With *V. commedens*, effects seem more localised and heterogeneous than with *H. fuscum*.

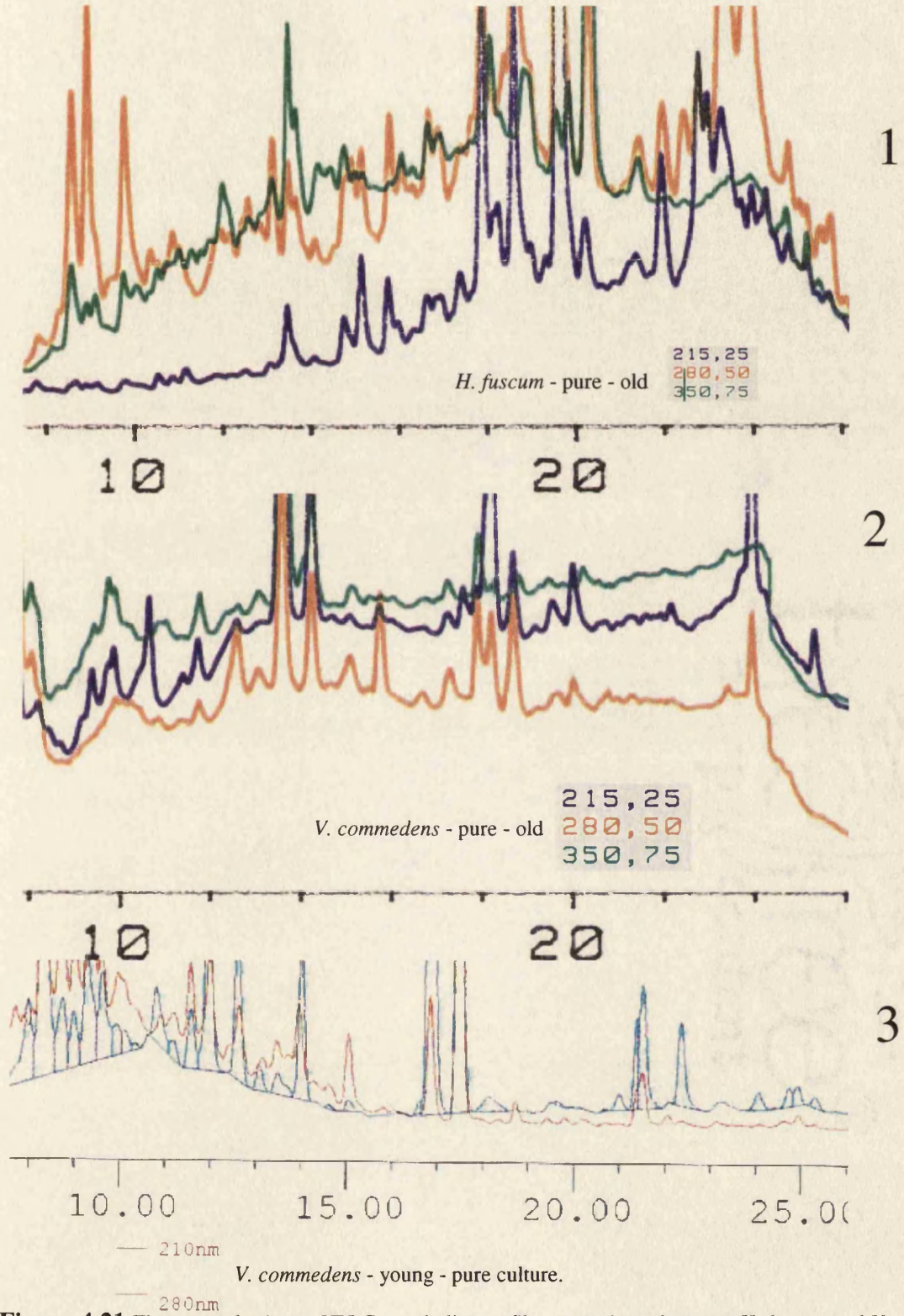


Figure 4.21 Three sample, 1-run HPLC metabolite profile comparisons between *H. fuscum* and *V. commedens* pure cultures and between different detection wavelengths. Retention times are displayed on the x- axis. (1) shows old *H. fuscum*, (2) shows old *V. commedens*, (3) shows young *V. commedens*. 280mn wavelength can be compared between each of the 3 spectra. (3) has many polar and few non-polar peaks whereas (2) has many mid-polarity metabolites and (1) has many polar and un-polar metabolites

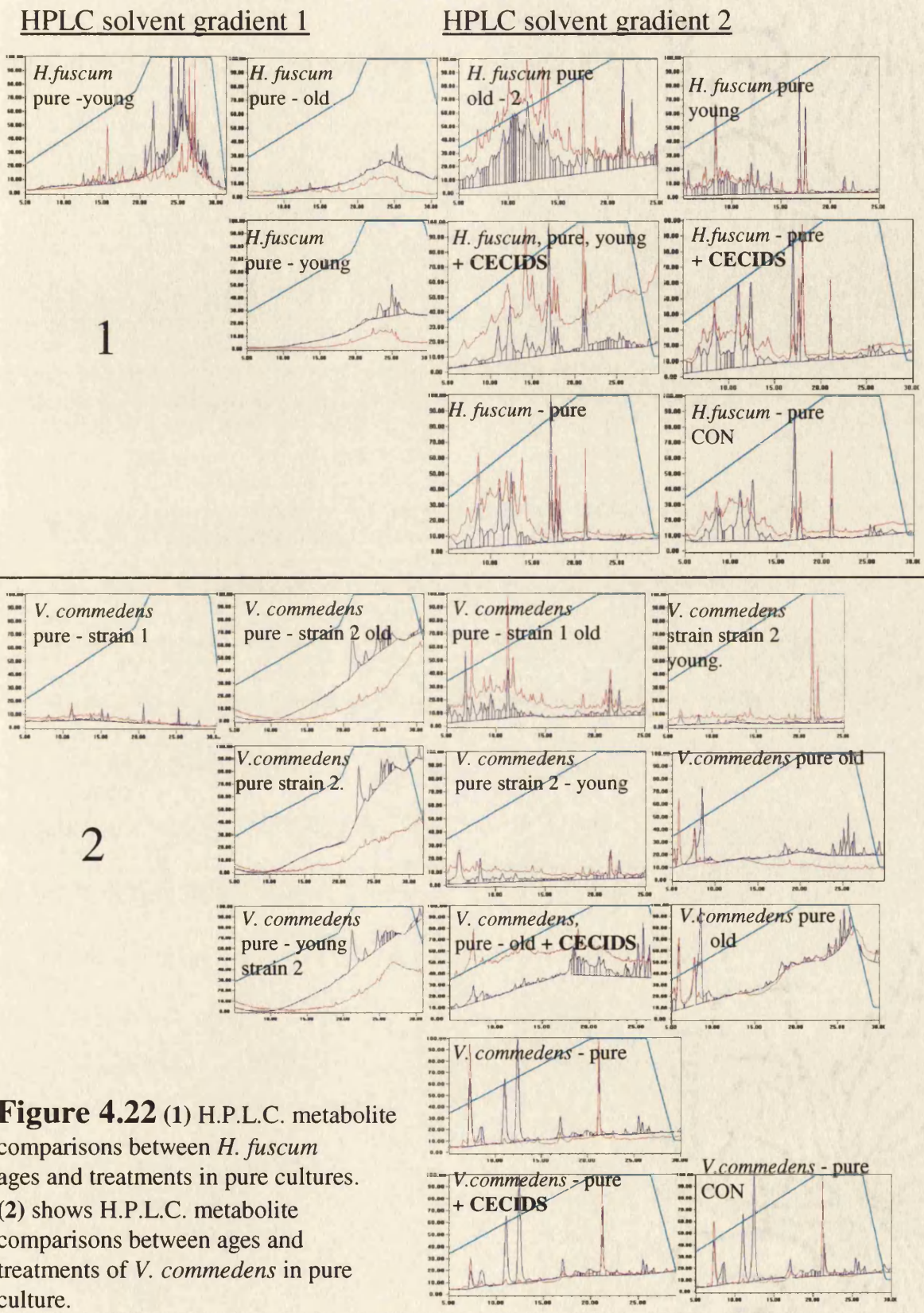


Figure 4.22 (1) H.P.L.C. metabolite comparisons between *H. fuscum* ages and treatments in pure cultures. (2) shows H.P.L.C. metabolite comparisons between ages and treatments of *V. commedens* in pure culture.

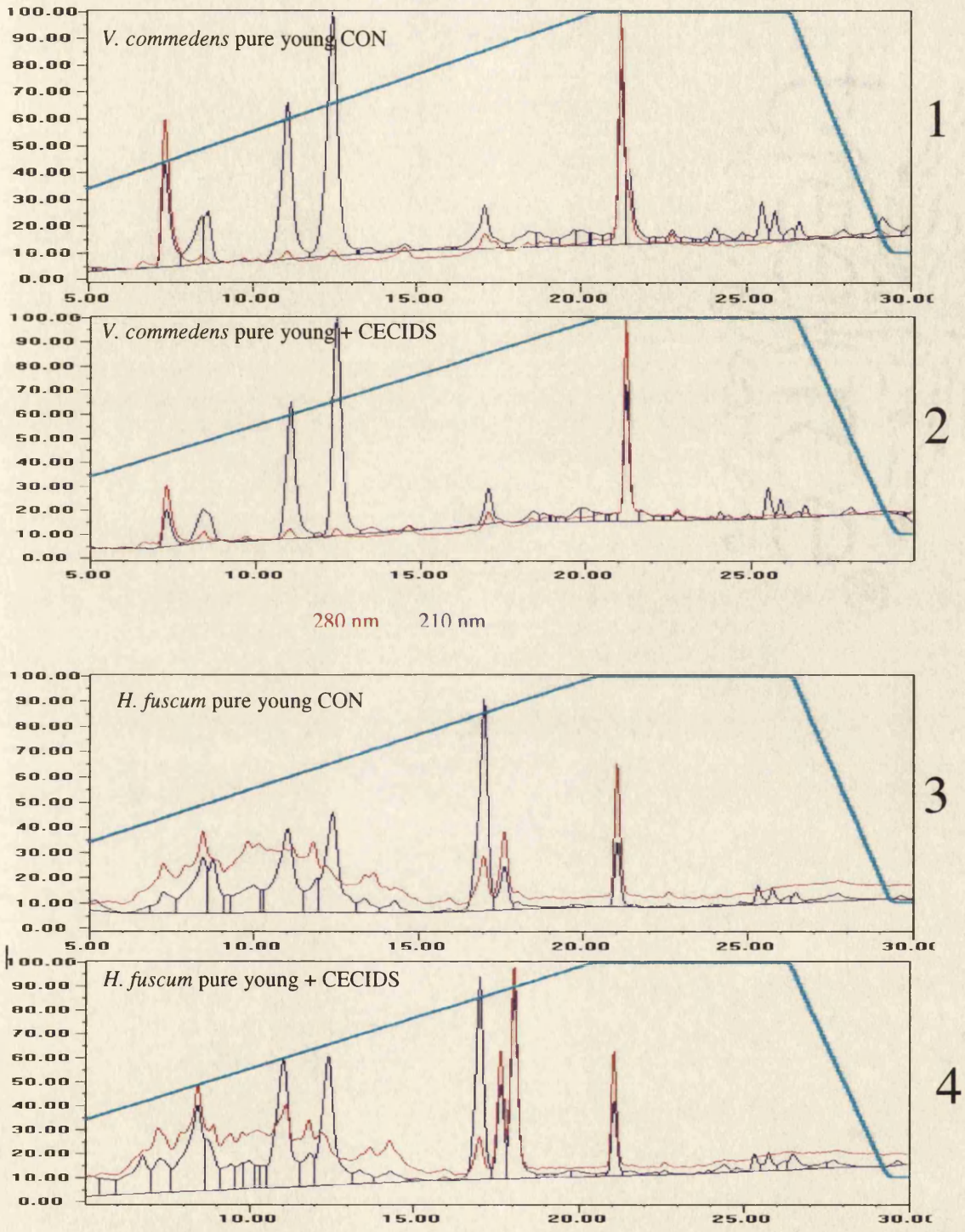


Figure 4.23 H.P.L.C. metabolite profile comparisons between *H. fuscum* and *V. commedens* at two wavelengths in the same run. (1) shows young *V. commedens*. (2) shows young *V. commedens* treated with cecids. (3) shows young *H. fuscum*. (4) shows *H. fuscum* treated with cecids.

to cecids with production of new polar compounds (at left-hand end of the profile). However, what was not expected was reduced production of *H. fuscum* non-polar compounds at the right-hand end of the profile.

Figure 4.22 (2) shows differences between solvent gradient systems, species, isolates and treatments of age and cecid larvae for *V. commedens*. At bottom left, using solvent gradient 1, can be seen the effect of age on *V. commedens* isolate 2, i.e. increased non-polar compounds in 210 nm wavelength spectra (as expected). Also can be seen the dramatic difference between the 2 isolates of *V. commedens* compared at the same age. *V. commedens* isolate 2 produces less polar and more non-polar molecules than *V. commedens* isolate 1. To the right of these profiles are shown those for solvent gradient 2. These show effect of cecids on old *V. commedens* isolate 1, i.e. increased non-polar compound production as expected. Two control comparisons are shown between young cultures of *V. commedens* isolate I and old cultures of *V. commedens* isolate 2 to show the extent of intrinsic metabolic heterogeneity within the cultures (Crowe 1997, Watkins 1998). There are differences and similarities, as with all comparisons, which indicates that not all of the observed changes above were due to the treatments alone, but a coupling of treatment-caused changes with instability-caused changes inherent to metabolic pathways in mycelial systems. The final single culture HPLC analysis figure 4.23 shows the effect of cecids on *H. fuscum* isolate 1 and *V. commedens* isolate 1 after 2 weeks of cecid interaction. In *V. commedens* there are no changes that could be said to be large enough to be attributed to cecid activity, at least not at this early-stage of interaction. However with *H. fuscum* there is already a notable extra peak produced at 18 minutes that is almost entirely absent from cultures without cecids.

4.5 Discussion

The results presented in this chapter demonstrate reciprocal changes induced by each living system on the other. This can be described in terms of developmental feedback. In general the results suggest that cecids are developmentally affected by changes in fungal environment (through ageing or species) through attunement in cecid life-cycle duration, numbers of offspring, and foraging pattern shapes of toroidal trajectories. In addition, results demonstrate that fungi are significantly affected by cecids via developmental alterations to pigmentation rate, insulation and oxidative ageing metabolism (Esser 1990). **At metabolic scales, the amount of hydrogen peroxide and peroxidase activity were reduced whilst the amount of chemical antioxidants increased within mycelia. Also the amount of catalase activity increased within peroxisomes inside hyphal compartments. In addition, free radical presence was shown to be most extensive at colony margins, which may explain why all but the smallest exploratory first-instar cecids were not found at colony margins of *H. fuscum*.** However, cecids of all stages were repelled to a lesser extent by colony margins of *V. commedens*. This suggests that cecid feeding at colony margins is tolerated by first-instar larvae more than mature mothers, which seem to behave as if they have become adapted to seek out the areas of fungal mycelia with most antioxidant activity, or insulation, and correspondingly most active translocation of fungal protoplasm (chapter 1). This partially contradicts and supports findings in chapter 3 that cecids seem to be located at the surfaces or boundaries of fungal domains within rotting wood. Previous researchers showed that cecids are most densely clustered at mycelial margins (Wyatt 1965) in mushroom houses. This apparent contradiction points to the possibility of an interesting relation between antioxidant availability, peroxidase-mediated decomposition of lignin, insulation of hyphae, protoplasmic-streaming in hyphae and effects of cecid induced perturbations to this system.

Compounds of similar structure are known to be produced in cecids and ascomycetes of *Hypoxylon* sp. These include a cecid metabolite called 2-hydroxy-5-3, 4,5-trihydroxy-phenly-cyclopentadienone, which may be harnessed as a precursor to the *Hypoxylon sp.* green quinone-like pigment called hypoxyxylerone (1, 3, 9, 11-tetrahydroxy - 6 - hydroxmethyldibenzo [b, h] xanthen - 8 - one) (Edwards *et al.* 1991). Not only is there a considerable similarity between several molecules known to be produced in cecids and ascomycetes but also a likelihood of reciprocal exchange, and metabolism of secondary compounds between wood co-habiting insects and fungi. This does not, however, necessitate similar metabolic function. Some evidence to support this suggestion comes from the work of Hashimoto and Asakawa who have reported a beetle-induced oxidation of the metabolic activity of fungal amides and cytochalasins into sesquiterpenoids in the ascomycete *Daldinia concentrica* (Hashimoto & Asakawa 1998). In this case it is thought that the dark pigment 4,9-dihydroxyperylene - 3, 10 - quinone, is a polymer which forms from oxidation of a melanin precursor called 3-methyl-3-4-dihydroisocoumarin, 5-methylmellin. This latter compound reacts with binaphtyl - a metabolite that is found in *Hypoxylon sp.* and *Daldinia sp.* (Hashimoto & Asakawa 1998) and may play a role in ascomycete pigmentation reactions.

Ergosterol, an insect hormone important in cecid paedogenesis (Hunt 1996) is similar in structure to binaphthyl. Perhaps ergosterol and other cecid compounds can alter the pigment metabolism of the chemically versatile *Hypoxylon sp.* ascomycetes. Similarly, it appears likely that fungal production of compounds like binaphthyl could affect cecid development. Work carried out by Hunt broadly confirms this suggestion (Hunt 1996). Some of the insect hormones that are necessary in controling insect progression through life-cycles may equally produce other effects in fungal mycelia such as increased hyphal insulation in *V. commedens.* **Introduction of compounds by cecids may have been directly or indirectly responsible for the novel peak at 18 minutes in the HPLC profile of *H. fuscum*** affected by cecids. Perhaps this novel compound could have been responsible for increased antioxidant production in *H. fuscum* mycelia. This kind of metabolic cycling of cause and effect creates possible developmental feedback loops in which antioxidants change development of fungi through pigmentation processes, in turn affecting foraging behaviour of cecid larvae as they develop into mother hemi-pupae. These reciprocal exchange ideas are supported by work by Hunt (1996), Ulrich (1943) and White (1977) that demonstrate ways in which alterations of cecid life-cycles occur with changes in chemical environment.

How relevant are these findings on pure cultures to the field distributions described in chapter 3? Colony margins in mushroom houses and in forests usually occur at the sites of interactions with other members of the local fungal community. As already demonstrated in chapter 3, cecids are found at PSP zones of interactions between species. Perhaps the biochemical metabolites that occur at the margins of expanding fungal colonies in single cultures are different from those that occur in interaction with other fungi. So the results of single culture experiments have improved our understanding but to a limited extent which, without previous fieldwork, would have led to some confusion. To improve our understanding of the biochemical ecology of these insects and fungi it was necessary **to relate cecid development to the properties of naturally occurring interactive mycelia**. This leads the way towards combined (interactive) fungal culture experiments - the topic of chapter 5.

CHAPTER 5: LABORATORY STUDIES OF INTERACTIONS BETWEEN CECIDS AND FUNGI FROM DECOMPOSING HAZEL WOOD, II: CECIDS ON INTERACTIVE FUNGAL CULTURES

5.1 Synopsis

Having considered developmental feedback that occurs between cecid larvae and single cultures of fungal mycelia in chapter 4, chapter 5 now applies essentially the same set of logic and laboratory methods to investigate more complex, field-relevant tripartite, interactive cultures of two or more fungal species and the effects that these fungal interactions have on cecid larvae. By the same token, reciprocal feedback effects of cecid larvae on either of the two interactive fungi and on the metabolic products induced by their fungal interactions are investigated.

5.2 Introduction

Insects, especially small mycophagous Diptera have not previously been observed with inter-specific fungal boundaries but such associations have been found with somatically incompatible intra-specific fungal interactions (chapter 1) (Boddy, Coates & Rayner 1983, Wyatt 1960 a, c). Spreading waves of mycelium were shown to influence the aggregation of cecids in mushroom beds (Wyatt, 1965) and in culture (Wyatt 1969). However, there has been little published work on the implications of dynamic fungal interfaces regarding reciprocal insect-fungal relations (Swift & Boddy 1984, Cooke & Rayner 1984, Rayner 1992 a,c), especially within decaying wood and bark. This is a notable omission, since it is the response to interaction with others of the environment that appears (as described in chapter 1) to sum up, in essence, the fundamental importance of fungal biology (Rayner 1993 a). It is then of prime importance in understanding cecid ecology, to study the relationship between cecids and fungi by culturing them in as near to their natural state as possible. This means using interactive combination cultures that produce a zone of reactive interface in which cecid activity and effects can be studied. Reactive interfaces between fungi comprise the constitutively added properties from both fungal partners and any other inhabitants of the interface zones. The chemico-physical properties that emerge from such interactivity may extend the ambit of such reactive zones. The combined chemical and physical conditions of such zones are expected to be unique to the organisms involved and how they relate to the environmental conditions that provide the arena in which this complex biophysical and biochemical performance takes place.

As outlined in chapter 1, there are four modes of response when two mycelial systems meet at their assimilative or explorative margins. The first three modes are intra-specific interactions whilst the last occurs with inter-specific reactions. Firstly, fusion of genetically identical somas (and subsequent maintenance of homokaryotic status) may occur. Secondly, somatically compatible fusions (mating) may occur between different homokaryon individuals of the same species. Surviving heterokaryons have two sets of different nuclei whilst only one type of mitochondria and peroxisome remain (Scazzachio 1987, Webber, Wakley & Pitt 1998) therefore forming at least temporary, if not long lasting and stable heterokaryons. Thirdly, somatic rejection occurs following fusion of two different homokaryon or heterokaryon mycelia of the same species.

Here conflicting instructions from different sets of nuclei and mitochondria and peroxisomes (Malik & Vilgalys 1999) cause fungal protoplasm to lose coherence and suffer the effects of oxidative-stress leading to senescent metabolic meltdown and hyphal apoptosis (Webber, Wakley & Pitt 1998). Fourthly, when members of different species meet, the formation of protective barrier mechanisms and pigments such as melanins may be induced in response to reactive oxygen species (ROI). A further response in this instance, with continued contact between different species, is the formation of a matted surface at the touching interface between mycelia. This material emerges from mass hyphal-senescence whereby initially deeply melanised mycelial domains become transformed in their chemico-physical properties into a hydrophobic pseudosclerotial plate (PSP). Once their formation is complete, such PSP zones often demarcate stable and long-lasting territories of mycelial domains within much rotting wood (Cooke & Rayner 1984, Rayner 1988 a, Boddy 1992). PSP is termed "spalting" by carpenters, who have used it to provide decoration to their furnishings for millennia. Often the structure of wood in advanced stages of decay consists of a durable, fragile, topologically mani-folded PSP lattice that is left behind once the majority of lignin has been removed by resident fungal-insect communities. Thus PSP contributes directly to the emergent cavity architecture of rotted wood structure in which insects and other invertebrates live. Indeed, PSP is occasionally the only remaining entity which maintains the integrity of the structure of rotted wood once the bulk of lignin has been removed.

We have already described the occurrence of a strong association between the dipteran cecid larvae *Brittenia fraxinicola* with PSP in chapter 3. These inter-specific ruderal interactions between pioneer fungal decomposers could hold a key to understanding the natural distribution ecology of *B. fraxinicola*, especially when involving the two fungi most associated with the field distribution of *B. fraxinicola* larvae, the ascomycete *Hypoxylon fuscum* and the basidiomycete *Vuilleminia commedens*. Extrapolating the concept that fungi produce increasingly durable synergistic inter-surfaces as their relatedness decreases, it comes as no surprise that interactions between species of fungi which are phylogenetically most separate, such as between species in completely different groups of Eumycotina, produce PSP at their interfaces of a type that is most strongly associated with the presence of this particular insect.

The purpose of the work described in chapter 5 was thus to examine the effect of *B. fraxinicola* on the emergence of PSP zones between *H. fuscum* and *V. commedens*. By the same token these investigations also asked what effect PSP zones and interactive fungi have on development of cecid foraging patterns and orientation behaviours. This concept of the foraging pattern being an integral part of an organism's development relates fungal, plant and insect development in parallel and allows for comparative study.

Figure 5.1 shows the different types of interaction pattern which emerged on plates inoculated with both the ascomycete *H. fuscum* and the basidiomycete *V. commedens,* as described. Figure 5.2 shows an interesting set of observations on emergent PSP zones between fungi using a combination of image analysis and physical imprinting of cecid trajectories through hydrophobic PSP-material residues that have stuck to the hydrophobic underside of plastic Petri dishes. The effects of cecid activity on PSP zone at the aerial interface can be seen in figure 5.3. The cecids flattened hyphae as they congregated and spread out over PSP and neighbouring fungal tissue, especially on the *H. fuscum* side of the border. Figure 5.4 shows the patterns created by cecid foraging trajectories cut through aerial mycelia of *V. commedens* and *H. fuscum*. Toroidal

foraging loops can be seen emanating from PSP zones, exploring into the culture media and returning back to the PSP zone.

5.3 Methods and materials

5.3.1 Experiments with interactive mycelia

5.3.1.1 Culturing systems

These were the same as in chapter 4 except when aseptically inoculating a combination culture plate, two 5 mm plugs of mycelium, each from a different species, were placed in the centre of a 9 cm diameter 2 % malt agar Petri dish, separated by a gap of 3 cm. Other patterns of inoculation were tried in attempts to develop a colony pattern that maximised the length of mycelial interface. These included surrounding a single central inoculation of one species with eight inoculations of the other species, and *vice versa* (Pattern 1). However, in practice, this was more effort than return, since the reactive interfaces produced were only marginally longer than in the former case. A simpler method was tried as a compromise. In this case one central inoculation of *V. commedens* was placed between two inoculations of *H. fuscum* 3 cm apart and slightly off centre to make a triangle orientation with *V. commedens* at the apex. It was found that this method worked best, so it was adopted for latter experiments (pattern 2). This inoculum orientation worked by initiating the emergent development of a curved and therefore longer reactive interface between the species. The reason *H. fuscum* had to have two inoculation plugs to achieve the curve was because *V. commedens,* being a Basidiomycete, grew at approximately twice the rate of the Ascomycete. There were two strains of each of *V. commedens* and *H. fuscum* (chapter 4). All interactive experiments used number 1 strains unless indicated. Those of strain 2 were more invasive than those of strain 1, and also more unstable (being heterokaryotic) in their development, both strain 2 species showed multiple-sectoring and highly variable growth. It was decided, for the purpose of determining cecid influence, to concentrate on the more stable fungal strains (strain 1).

5.3.1.2 Computer image analysis

This was as in chapter 4 except that the fungal cultures were grown for a week before being treated with cecids (unless otherwise specified in the results section 5.4).

5.3.1.3 Positions of cecids in interactions, area mapping with acetate sheet

To monitor positions of cecids within culture agar and black PSP zones, and to map the areas of PSP zones using acetate sheets, a strong heat-free fibre optic light source was used to shine through culture plates. Any cecids within the PSP zone were cast in shadow, and the demarcation of PSP could be seen more clearly and traced with a marker pen onto the acetate sheet that was taped to Petri dish undersides. When area mapping, the shapes corresponding to different developmental zones were cut out of the acetate and weighed. These weights were converted to area by dividing by the weight of a single cm^2 of acetate. An alternative method for counting cecids within cultures was to dissect the entire agar surface by cutting into 1 cm^2 squares and keeping a tally of the number of cecid larvae found whilst dissecting in each location. The former method had an advantage in that the plate could be monitored over time rather than just once. The latter method's advantage was that no cecids, of any size, were left un-counted by being obscured behind deeply pigmented agar. Four experiments were carried out using the acetate mapping technique as follows:

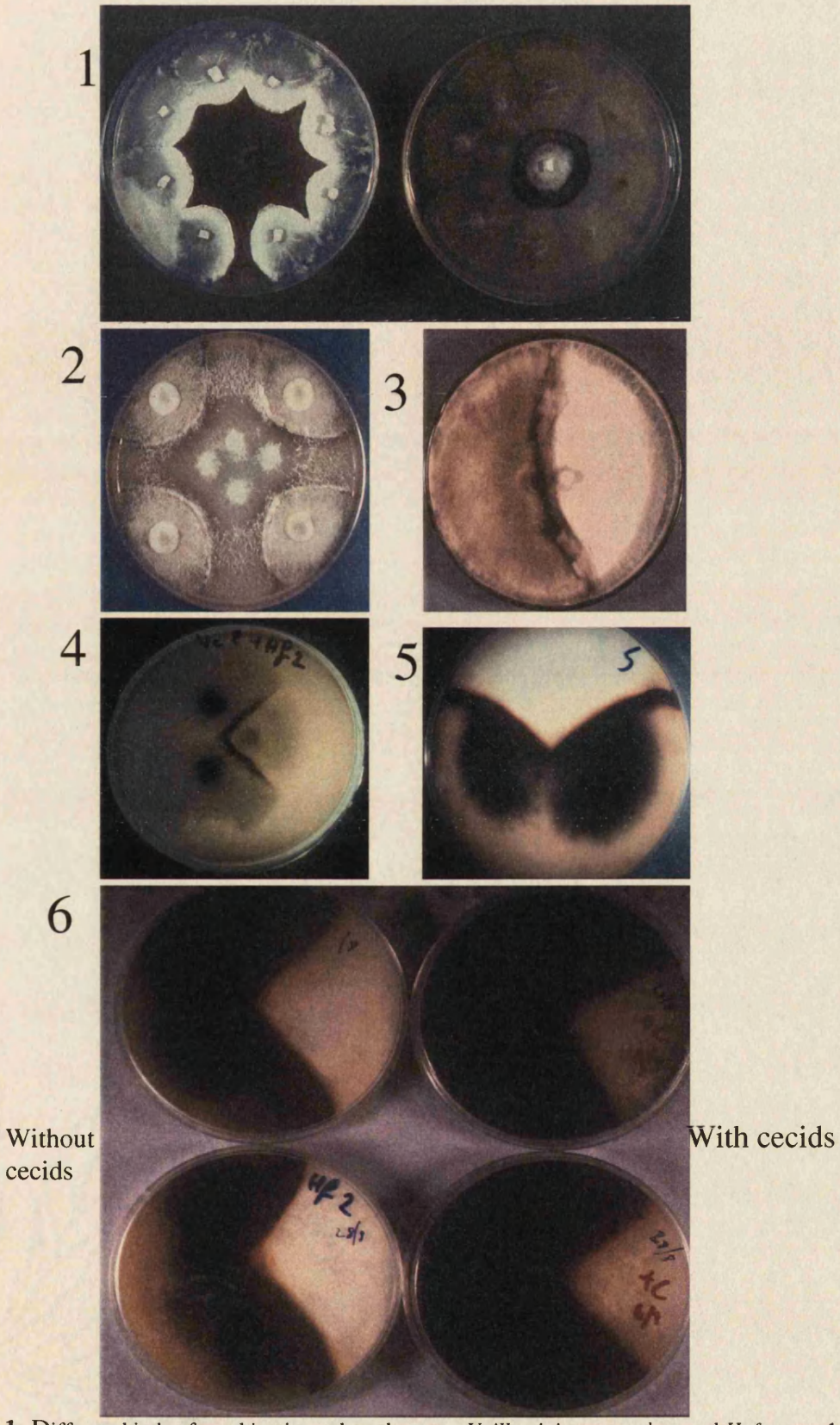


Figure 5.1 Different kinds of combination culture between *Vuilleminia commedens* and *H. fuscum*. 1, top left; patterns with one central inoculum and eight around the edge which maximise boundary zone areas for the study of PSP zones.2; a simpler version using 4 equal numbers of inoculum plugs. 3; a PSP zone is hidden under up-thrusting of aerial mycelium. 4; a young culture pigments from the centre outwards, 5; an older culutre of the same type is by now heavily pigmented; 6 an experiment to show the effect of cecids (at right) on the development of PSP zones.

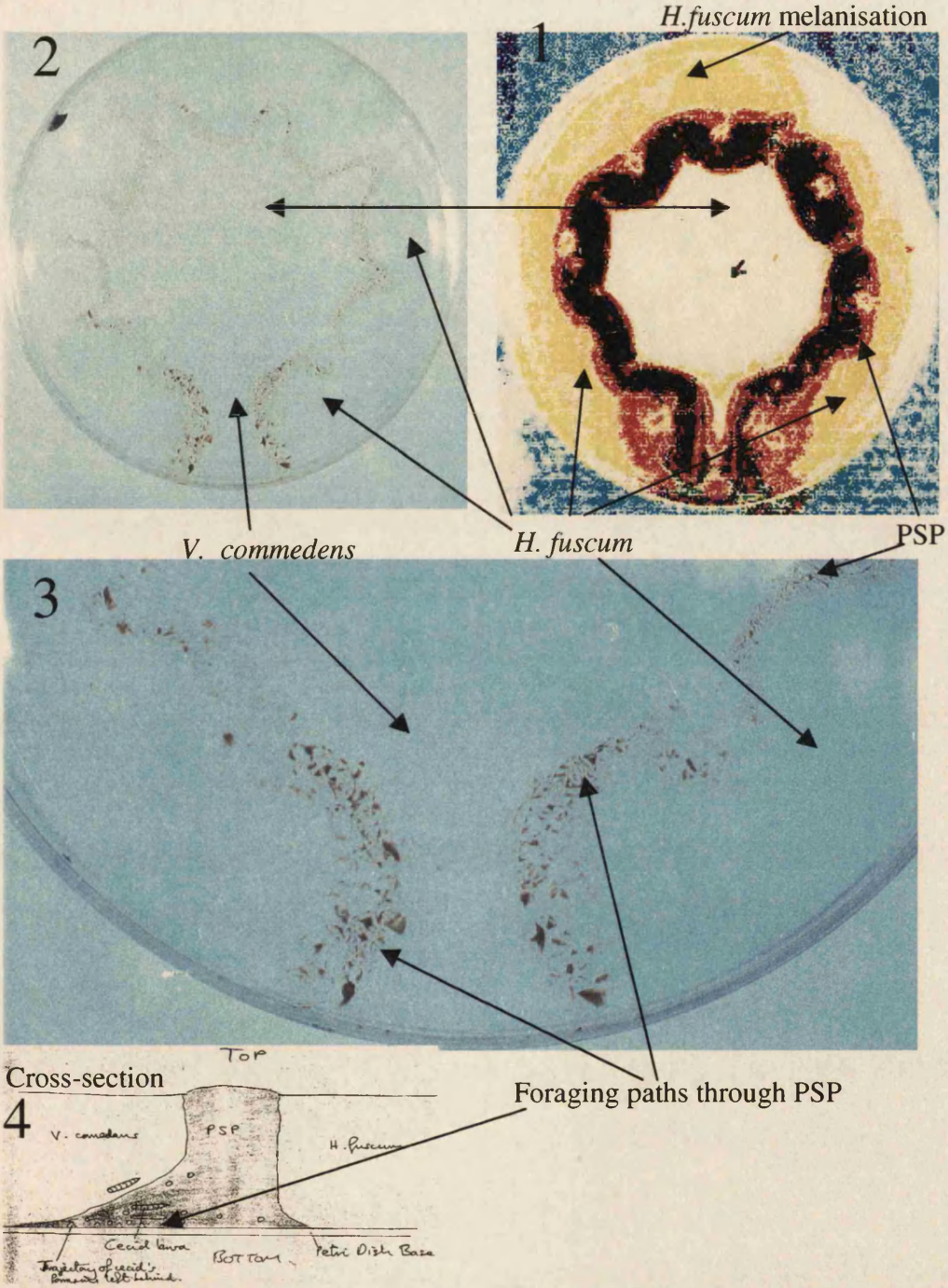


Figure 5.2 Problems in overcoming the difficulties of observing darkly pigmented boundary zones can be overcome with image analysis (in this case image enhanced photograph) shown as 1. This image reveals contours in the melanisation and PSP not clearly visible beforehand (see previous figures). A simpler method is to remove agar from the petri plate and observe trajectories of *Brittennia fraxinicola* foraging pathways through the PSP which remains stuck to the petri-dish (2 + 3). The trace, faithful to the PSP zone, could only be achieved with cultures treated with cecids. 4 shows a drawing from laboratory notes illustrating this phenomenon is cross section with cecids cutting long-lasting pathways under hydrophobic PSP precipitates.

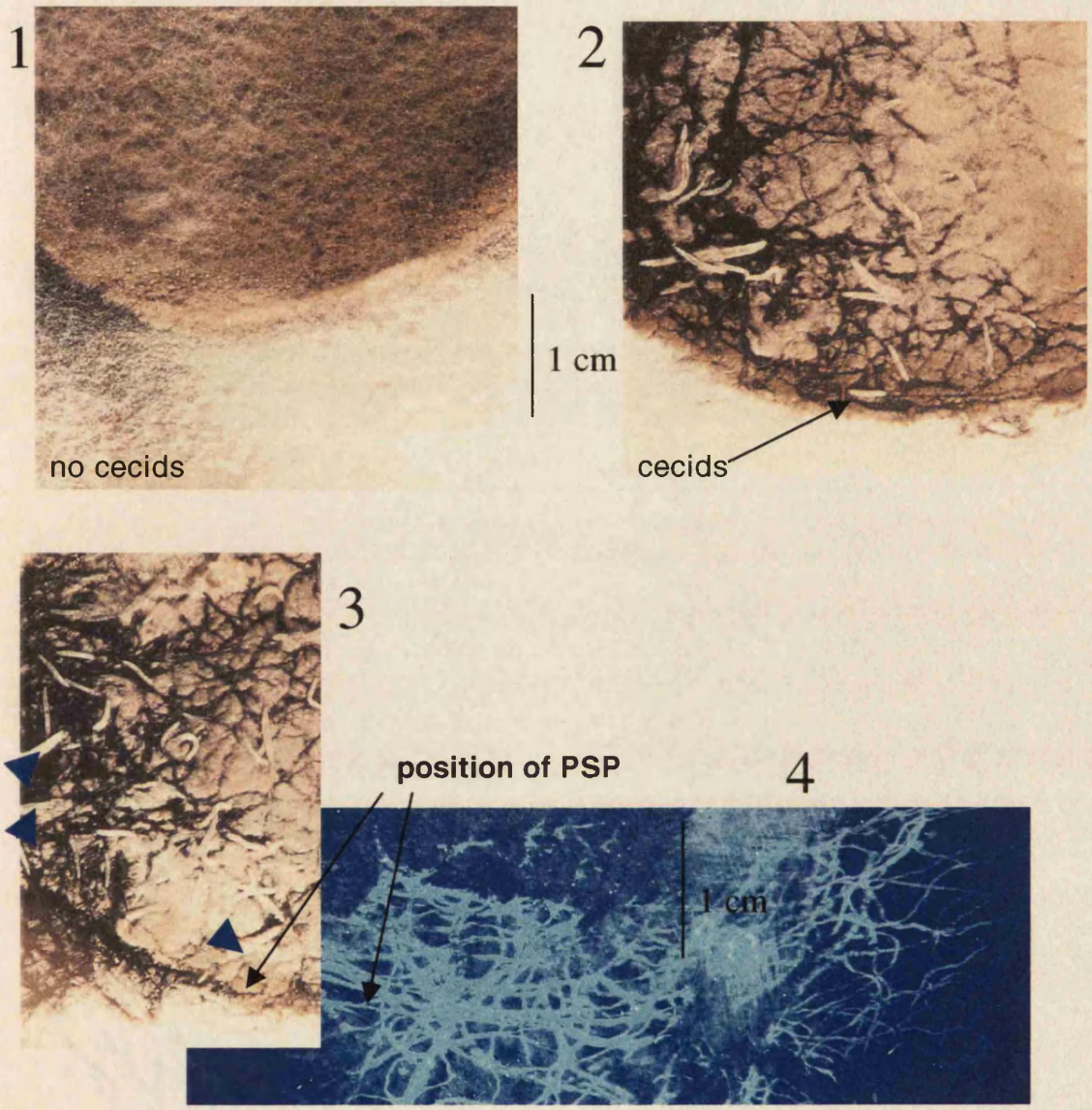


Figure 5.3 (1) and (2) show effects of insects on combination cultures; (1) top left an interaction interface between *H. fuscum* and *V. commedens* without cecid larvae. (2) shows treatment with cecids. Aerial mycelium has been flattened by foraging pathways, exposing underlying PSP boundaries of thicker development.

(3) and (4) show effects of combination cultures on insects; (4) shows traces of foraging trajectories left after peeling away the interface region. Loops can clearly be seen in this PSP. A surface view (3) is orientated to show position of PSP zone underneath. Large mother larvae can be seen close to the PSP zones (arrows).

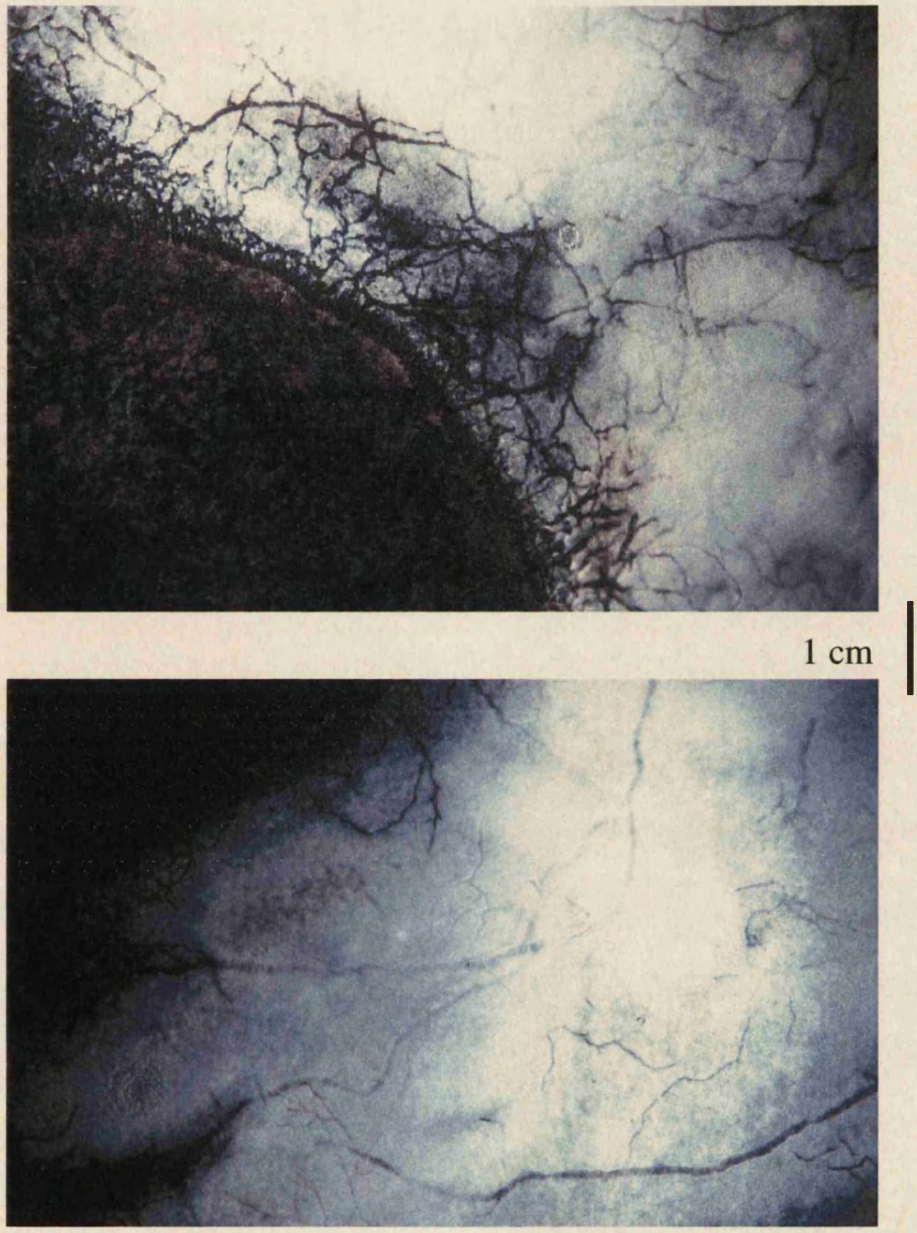


Figure 5.4 Foraging trajectories of *Brittennia fraxinicola* on combined cultures of *H. fuscum* and *V. commedens*. Top shows more of the trajectory patterns in *H.. fuscum* and at the PSP boundary. Below are trajectories found in *V. commedens* as well as some at the PSP boundary. Foraging loops with measurable radii can clearly be seen in both images, with smallest loops just on the interface between PSP and *V. commedens*. Foraging loop radii can also be seen increasing with distance from PSP zones.

5.3.1.3.1 Experiment 1

The first experiment was a one-month temporal mapping of interactive areas of inoculation pattern 1, treated with and without cecids. Emergence of areas was regressed over time on the x-axis. Paired t-tests were calculated to compare sample similarities (n>10). Cecids were added at day zero, the same day as inoculation of the fungi into Petri dishes. Experiment 1 was then repeated with pattern 2, but in this case the areas were only measured once after six days and treatments were compared with a Mann-Whitney test (n=4). In this second version of the experiment, the cecids were added three days after the inoculation of fungi, thus the cecid treatment in this case was only 72 hours in duration.

5.3.1.3.2 Experiment 2

The second acetate mapping experiment used inoculation pattern 2 with a timecourse of one month's duration in which cecid larvae were added to treatments after seven days and mapping took place twice on days 11 and 24.

5.3.1.3.3 Experiment 3

The third acetate mapping experiment used inoculation pattern 2 with a timecourse of one month in which the cecids were added on day 18 and mapping took place on day 30.

5.3.2 Tissue-lysis printing

Tissue prints for antioxidants (DPPH), H_2O_2 production, peroxidase and catalase enzyme activity were developed from one month interaction cultures with and without *B. fraxinicola* larval treatment. Methods used were identical to those of chapter 4, except that interactive cultures were used. One novel tissue print for total protein was also added.

5.3.2.1 Total protein assay using Coomassie Blue tissue print

Following the strategy developed for tissue-lysis printing in chapter 4, a new method was added for the development of stains to show total protein produced across different zones of interactive mycelial cultures with and without cecids. Two reagents were used in this blue stain tissue print for total protein localised on nitro cellulose membrane. The first reagent was a stain and the second a de-stain. The two reagents were prepared as follows. Staining solution was a mixture of 0.1 % "brilliant blue R" (Coomassie Blue) [- Sigma chemical B0149] (0.1g in 100 ml), 20 % methanol (20 ml in 100 ml) and 7 % acetic acid (7 ml in 100 ml). The solution was made up to 100 ml with milliQ water and placed in a labelled trough. De-staining solution was made with a mixture of 7 % acetic acid (21 ml in 300 ml) and 50 % methanol (150 ml in 300 ml), made up to 300 ml in milliQ water and placed in another labelled developing trough. A third trough was filled with pure milliQ water for rinsing between stains. The protocol was to take a fresh nitro cellulose tissue print (as in chapter 4) with forceps and immerse into staining solution for 1 minute, then to immerse in MilliQ water whilst swirling, and finally to immerse in de-staining solution and gently agitate for several minutes. The Coomassie Blue colour only remains bound to regions where it has been fixed into the protoplasmic protein that was already bound to the nitro cellulose membrane. A photograph was taken of the developed print after blotting on clean tissue.

5.3.3 HPLC analysis

The same high-pressure liquid chromatography (HPLC) methods were employed as in chapter 4. Three HPLC runs were made to separate fungal metabolites. Samples were used from three different zones of fungal interactivity, *H. fuscum*, PSP zone and *V. commedens*.

5.3.4 Foraging loop investigations

Foraging loop radii were recorded in mm for inter-specific interactive cultures between *H. fuscum* and *V. commedens* and also interactions of two intra-specific somatically incompatible isolates of the basidiomycete *Coriolus versicolor*.

5.3.5 Interactive observations

A light source was used to shadow-cast the topography of interactive cultures to see the effect of cecids on features such as height of a PSP zone ridge which developed in older interactive cultures, and the degree of PSP zone cracking, development of blisters in or on either side of PSP zones.

5.3.6 Statistical analyses

For sample sizes greater than 9, the t-test was used (if $n<9$ the Mann-Whitney test was used) to test for differences between cecid treatments and controls (Wheater & Cook 2000). Regression analysis was performed over time to follow the development of interactive zones in experiment 1. Fisher's Exact test was used on frequency data regarding observations of interactive cultures (used instead of Chi-squared test when expected values are < 5) (Howitt & Cramer 1997). Correlation analysis was carried out between related factors on scatter plots where the dependence of one variable on the other could not be determined (Wheater & Cook 2000).

5.4 Results

5.4.1 Foraging loops on interactive cultures

Measurements of foraging loop radii are shown in figure 5.5. There were highly significant differences between treatments using t-tests ($P<0.0001$), the print-outs of which can be found in Appendix 7. Loop radii were also measured using interactions between somatically compatible and incompatible strains of the same fungus species *Coriolus versicolor*, on which *B. fraxinicola* also survived in laboratory conditions. Measurements of foraging loops on antagonistic intra-specific interactions were shown to be significantly different ($P<0.01$) in zones of somatic incompatibility, which produced senescent dark brown hyphae but no PSP as in figure 5. 6.

5.4.2 Larval positions and interactive cultures

The positions of *B. fraxinicola* larvae in different parts of interactive cultures between *H. fuscum* and *V. commedens* following dissection of culture plates after one month are shown in figure 5.6. Cecids were observed in tight clusters within agar, especially within the PSP zones. The differences between dissected cecid populations in different parts of the plate can be seen to be highly significant through use of the t-test with critical value of 1.96 ($P<0.0001$). Data from dissection of entire plates into 1 cm squares was different from that obtained by just counting the numbers of cecids on the culture surface of 1-month-old plates, as

shown at the top (1) of figure 5.7. However, the PSP zone was much smaller in area than either of the mycelial domains. To make the test valid, data were adjusted into concentrations of cecids found on surface mycelium in each location, as shown at figure 5.7 (2). In the latter case, there is again a highly significant maximum density of cecids at the PSP zone surface ($P < 0.005$). The effect of age made little difference to the latter result, although, after three months, the surface cecid populations were significantly highest on *H. fuscum*, whilst the concentrations of larvae were now significantly different between all three zones as shown in figure 5.8, with maximum cecid concentration residing on PSP zones. The linear relationship between thickness of PSP at the surface and at the base is not a 1:1 ratio. From the gradient of the slope we can tell that PSP dimensions at the surface of the agar increase 1.5 times faster than the dimensions of PSP at the base of the agar. There is a linear 1:1 inverse relationship between the areas of *V. commedens* and *H. fuscum*. However, the relationship between the two species areas, and the area of emergent PSP is more complex in the form of two inversely proportional and intersecting polynomial curves (bottom graph figure 5.8). Reading the graph along the x-axis, when PSP is low, areas of *V. commedens* are highest and areas of *H. fuscum* are at their lowest. As the amount of PSP increases, the areas of both fungi become equal at about 29 cm^2 for each colony. The area of PSP continues to rise in circumstances when there is more *H. fuscum* than *V. commedens*, giving rise to PSP areas of about 10 cm^2. However, maximum sized PSP areas were achieved when the proportions of fungi were approximately equal. Figure 5.9 shows pictures of plates with different areas of mycelial domain and PSP zone. Figure 5.10 shows light being passed through PSP from the fibre-optic light source. The distinction between *H. fuscum*'s melanised non-PSP mycelium and darker, sclerotised PSP zone is clearly visible using this technique.

5.4.3 Interactive fungal area results

Cultures from which measurements were made by tracing areas onto acetate sheet are shown in figure 5.11 for both those treated with *B. fraxinicola* larvae and non-treated controls. Results of temporal analysis of plates with developing PSP interfaces emerging from initial inoculation pattern of figure 5.11 (7) show a significantly increased ($P<0.05$) emergence of PSP in cultures treated with cecid larvae (figure 5.12). In control cultures there was a trend for the area of melanised regions of *H. fuscum* mycelium to increase as the mycelium aged (as with pure cultures seen in chapter 4). However, when cecid larvae were present, this melanised area was reduced compared to cultures without cecids. This latter melanisation trend is, however, not significantly different when using the t-test on grouped data ($P>0.05$). To see Minitab print-outs of this t-test analysis, please see Appendix 8. Figure 5.13 shows the results of a small experiment to see if results were changed by the initial inoculation pattern. This experiment used the initial inoculation pattern shown in figure 5.11, (3). Despite small sample sizes, a Mann-Whitney test for non-parametric data showed significantly greater PSP and distances from developing mycelial domains to the edge of the Petri dish ($P<0.05$), for plates treated with cecid larvae. Also, in this experiment, the extent of *V. commedens* growth was severely reduced by the presence of cecids, whereas the difference made to area of *H. fuscum* by cecid activities was minimal. Figures 5.14 and 5.15 show similar results for repeats of experiments 2 and 3 using the initial inoculation pattern shown in figure 5.11 (7). Experiment two was measured at two times, whereas the data of experiment 3 was accumulated once at the end of a 30-day time course.

5.4.4 Computer image analysis results

The following results show what can be achieved with computer-aided image analysis. Figure 5.16 shows computer image analyses for 6 Petri dishes containing interactive cultures of *V. commedens* and *H. fuscum* treated with and without cecids as viewed from the top. Compilation graphs in figure 5. 17 show data from top (1), underside (2) and membrane controls (3 and 4). Radar plots are useful visualisations of data variability. Radar plots for amalgamated results for all available data are shown in figure 5.18. The significance of amalgamated data is shown graphically in figure 5.19, which demonstrates high significance ($P<0.001$) of results with high sample sizes ($n=37$) tested with t-test. Cecids are shown to increase PSP and decrease both *H. fuscum* and *V. commedens*. The greatest effect of cecids was to increase the area and rate of emergence of PSP zone. A minitab statistical print out of image analysis t-tests can be seen in Appendix 9.

5.4.5 Interactive culture observation results

Figure 5.20 shows the growth of a third fungus species introduced via cuticles of *B. fraxinicola* larvae from the field. Also shown is the potential for PSP to physically disintegrate in ageing agar plates and cause large cracks to occur. Figure 5.21 shows an interactive culture using *V. commedens* strain 2. Figure 5.22 shows results of ranked frequency descriptions of such fungal cultures (graph) and radar plots of percentage overlap between descriptions for the same plates. Again the radar plots provide an excellent way to visualise large amounts of complex data. Shaded areas link factors most strongly overlapped in terms of the percentage of plates described where points are most extended. For example, at top right, of all the plates which possessed invading *V. commedens* wave fronts, 20 % were contaminated with third-party fungi, 15 % had lifted PSP zone with bubbles underneath, <10 % had emergent PSP ridges or any sharp PSP demarcation, and 30 % had a diffuse PSP demarcation. Table 5.1 shows this percentage information in a table. Also shown are Fisher's Exact tests of the significance of these descriptive relations (Howitt & Cramer 1997). The 4 significant results are shown with an asterisk, linking PSP cracking to presence of third-party secondary colonising fungi and the emergence of PSP ridges that consisted of a small raised bevelled edge that ran parallel in direction and centrally within PSP zones. Emergence of PSP ridges was significantly linked to the emergence of lifted pockets of PSP zone with bubbles of gas underneath, as well as to PSP crack formation. *V. commedens* invasion wave fronts into *H. fuscum* domains were significantly linked to the emergence of PSP with ridges. Statistical output from SPSS for Windows which shows Fisher's Exact tests amongst other cross tabulated data is shown in Appendix 10.

Figure 5.23 shows graphs that demonstrate the differences in PSP width between the culture's agar surface and the base of the Petri dish, with and without cecid treatments. Although the effect of cecids can be seen to have increased the width of PSP in contact with air and at Petri dish undersides, only the trend at the surface was significant. In both sets of data the width of surface PSP is significantly greatest. The graph at the bottom shows that combination culture plates inoculated with *V. commedens* isolate 2 and treated with cecids possessed significantly less *V. commedens* invasions into *H. fuscum* domains than *V. commedens* isolate 2 without cecids. Also the graph shows that the incidence of third-party secondary colonising fungi can be attributed to the treatment with *B. fraxinicola* larvae and not to culturing contamination.

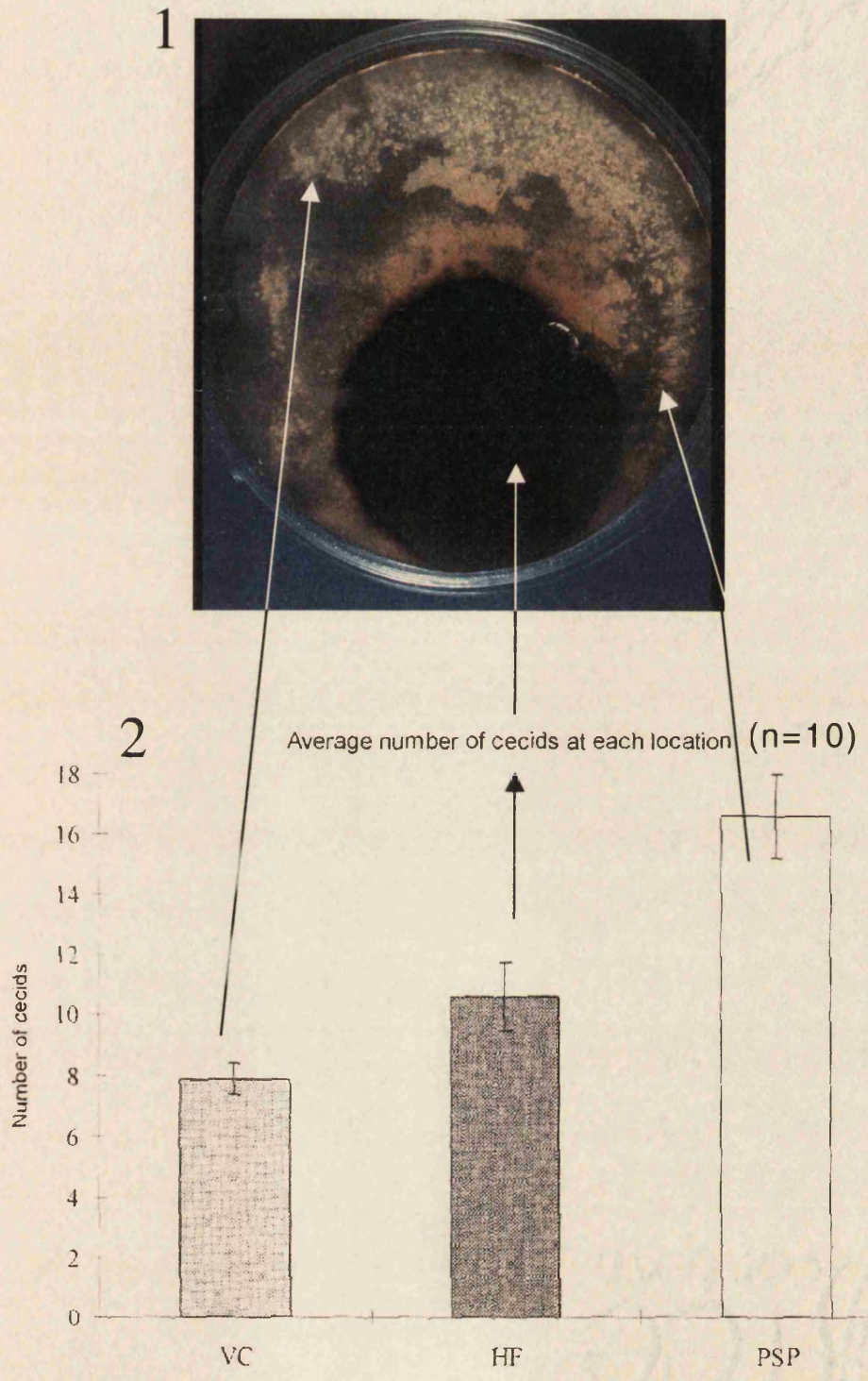
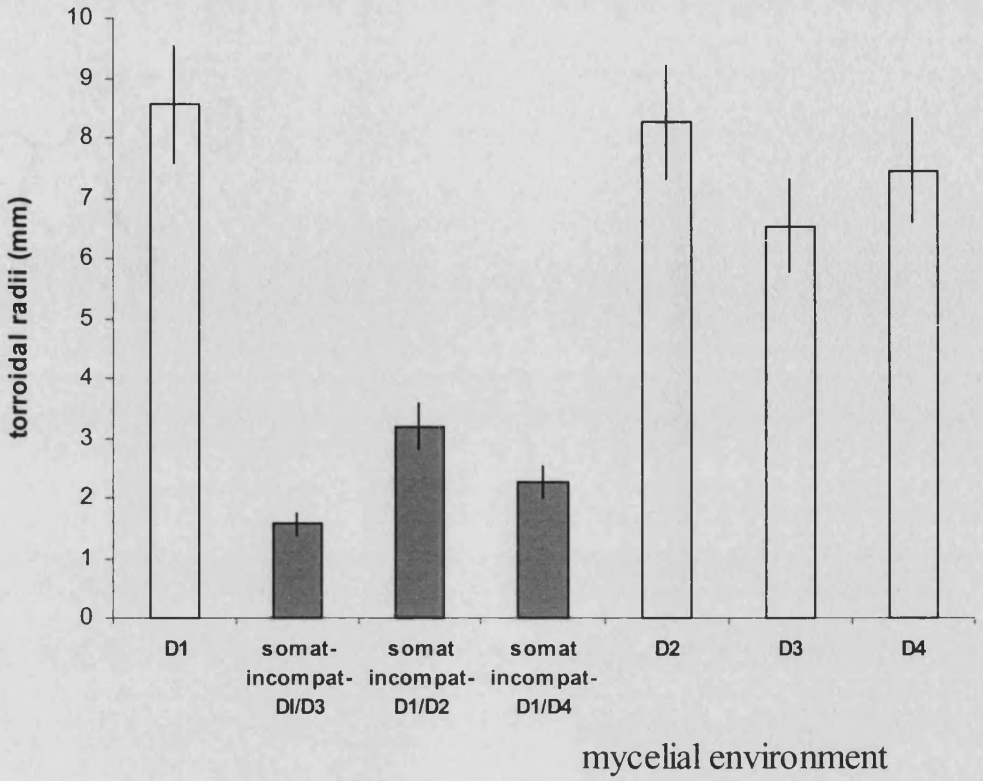


Figure 5.5 Positions of cecids on combination cultures. (1) shows a plate before dissection. (2) shows mean numbers of cecids found in each area after dissection (n=10).

Key: VC = *Vuilleminia commedens*, HF= *Hypoxylon fuscum* and PSP = pseudoscelerotial plate zone. Student t-tests show significant different cecid populations ($P<0.0001$) between the 3 fungal domains.

1

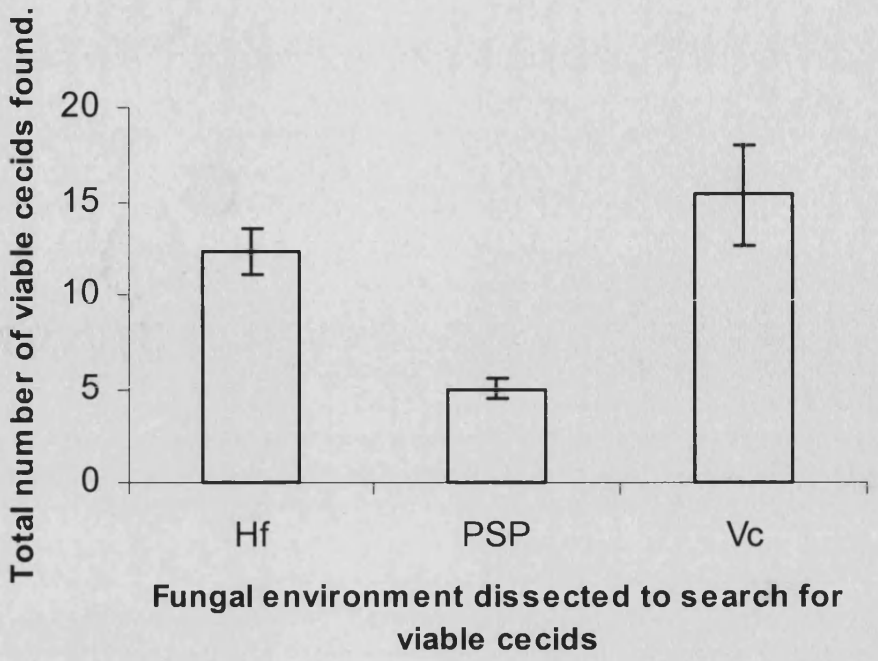


2

T-test							
P. nos	D1	somat incompat-DI/D3	somat incompat-D1/D2	somat incompat-D1/D4	D2	D3	D4
D1	1	**0.001**	**0.001**	**0.002**	**0.007**	**0.001**	0.267
SI-DI/D3		1	**0.001**	**0.0001**	**0.0001**	**0.005**	**0.0001**
SI-D1/D2			1	0.425	**0.0001**	**0.011**	**0.0001**
SI-D1/D4				1	**0.001**	**0.032**	**0.0001**
D2					1	**0.0001**	**0.001**

Figure 5.6 (1) shows cecid foraging loop radii, shown increasing on the y-axis, and type of *Coreolus versicolor* strain or zone of inter-species somatic incompatibility on the x-axis. A table of t-test probability numbers (2) between the data stets (n=30) confirms significant difference ($P<0.01$) between zones of somatic incompatibility (1 - grey) and mycelia (1 - yellow). **Key**:P.nos = probability, D1, D2 ect. = strain 1, strain 2 ect., somat incompat-D1/D3 = zone of intra-specific somatic incompatability between strain 1 and 3 of *C. versicolor*.

1



2

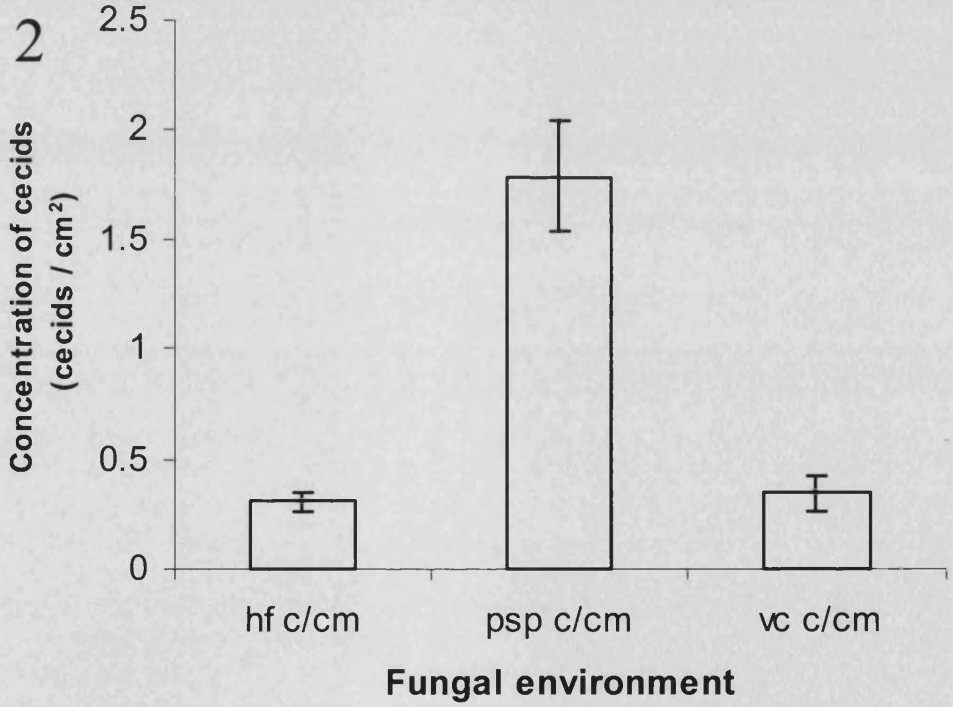


Figure 5.7 (1) shows mean numbers of field cecid larvae found in different zones of combination cultures (2) shows mean density of cecids found in each cm^2 (c/cm) of each zone. Significance was Mann-Whitney tested (n=6). Standard error bars are shown. (1) shows that whereas total numbers of cecids in PSP zones are significantly lower ($P>0.005$) the density of cecids in PSP zones is significantly higher ($P<0.005$) than in mycelia on either side of PSP zone.

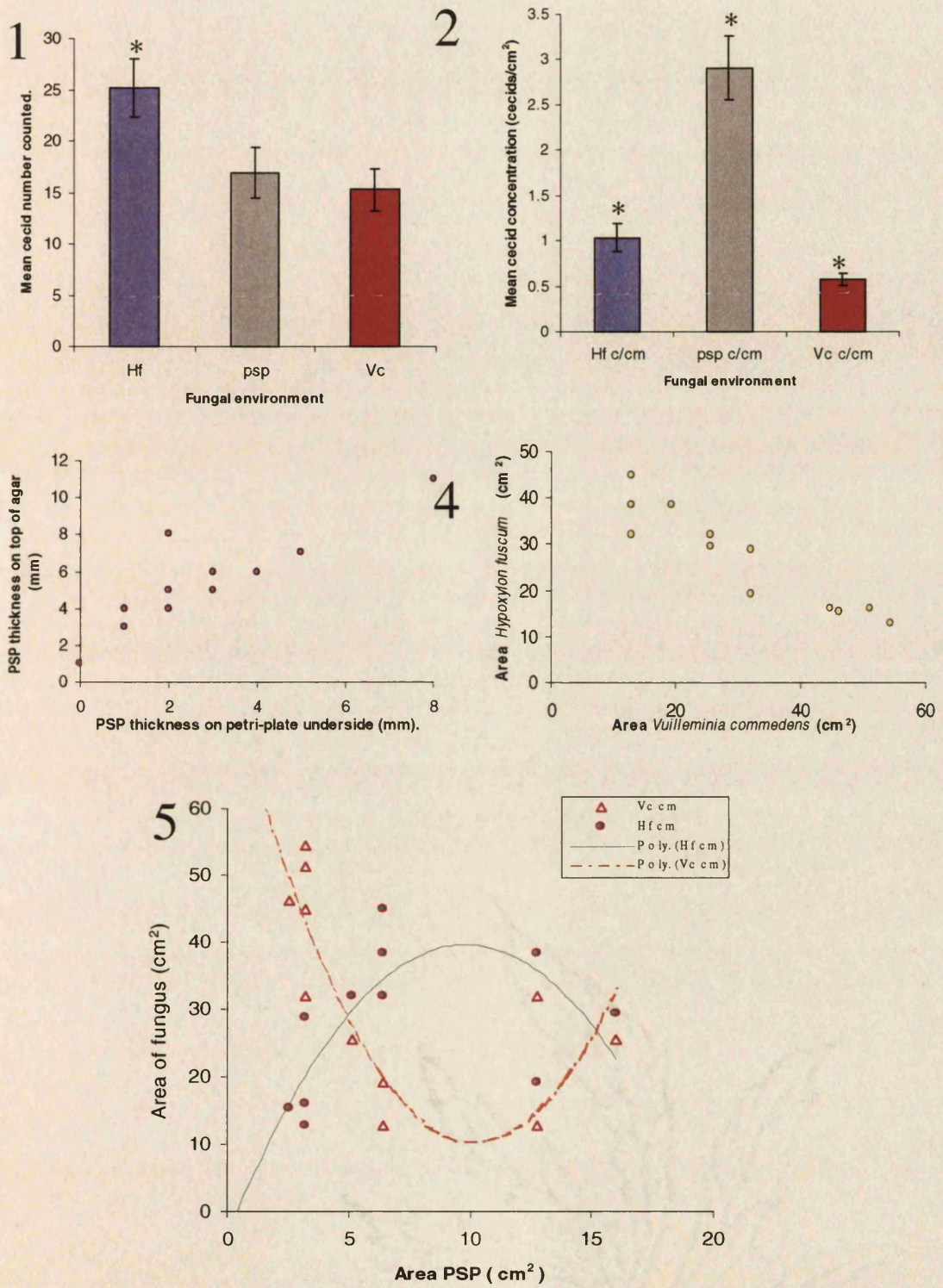


Figure 5.8 Asterisks (*) show significant difference (P<0.05). (1) shows mean numbers (n=23) cecid larvae found in different zones of interactive culture, (2) shows mean concentrations (n=23) of larvae (larvae/cm^2) in different interactive zones. (3) shows relationship between PSP thickness on top and bottom of agar after 3 months of interactive development (P<0.001). (4) shows relationship between areas of *V. commedens* and *H. fuscum* in combined cultures (P<0.0001). (5) shows two parabolic functions fitting the relationship between PSP area at surface and the corresponding areas of *H. fuscum* and *V. commedens* in interactive cultures.

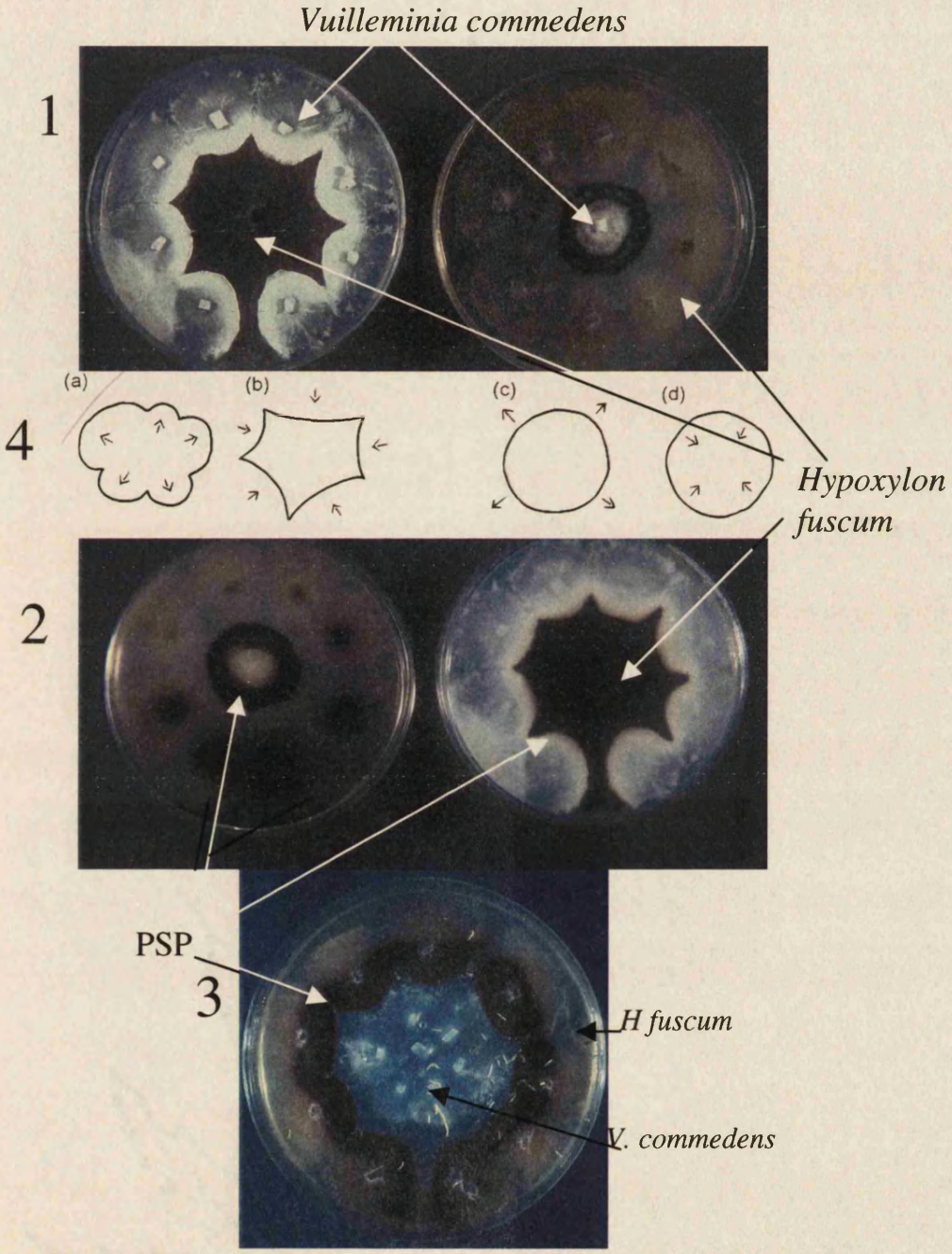


Figure 5.9 Designs of combination cultures between *Hypoxylon fuscum* and *Vuilleminia commedens* to examine the effects of combination cultures on cecids and the effects of cecids on the combinations of fungi and the pseudo sclerotial plate (PSP) zone. 1 shows the top view of two ways of inoculating the fungi to maximise interactive boundary distance. 2 shows the underside of these same cultures where hydrophobic PSP zone precipitates can be seen annealing to the plastic petri-plate-undersides. 3 shows cecid larvae foraging over a similar culture. 4 shows , from shapes, whether boundaries are likely to be expanding or contracting; a) + b) shapes indicate probable direction of movement. c) remnant domain, d) disturbance domain. (from Foreman 1995)

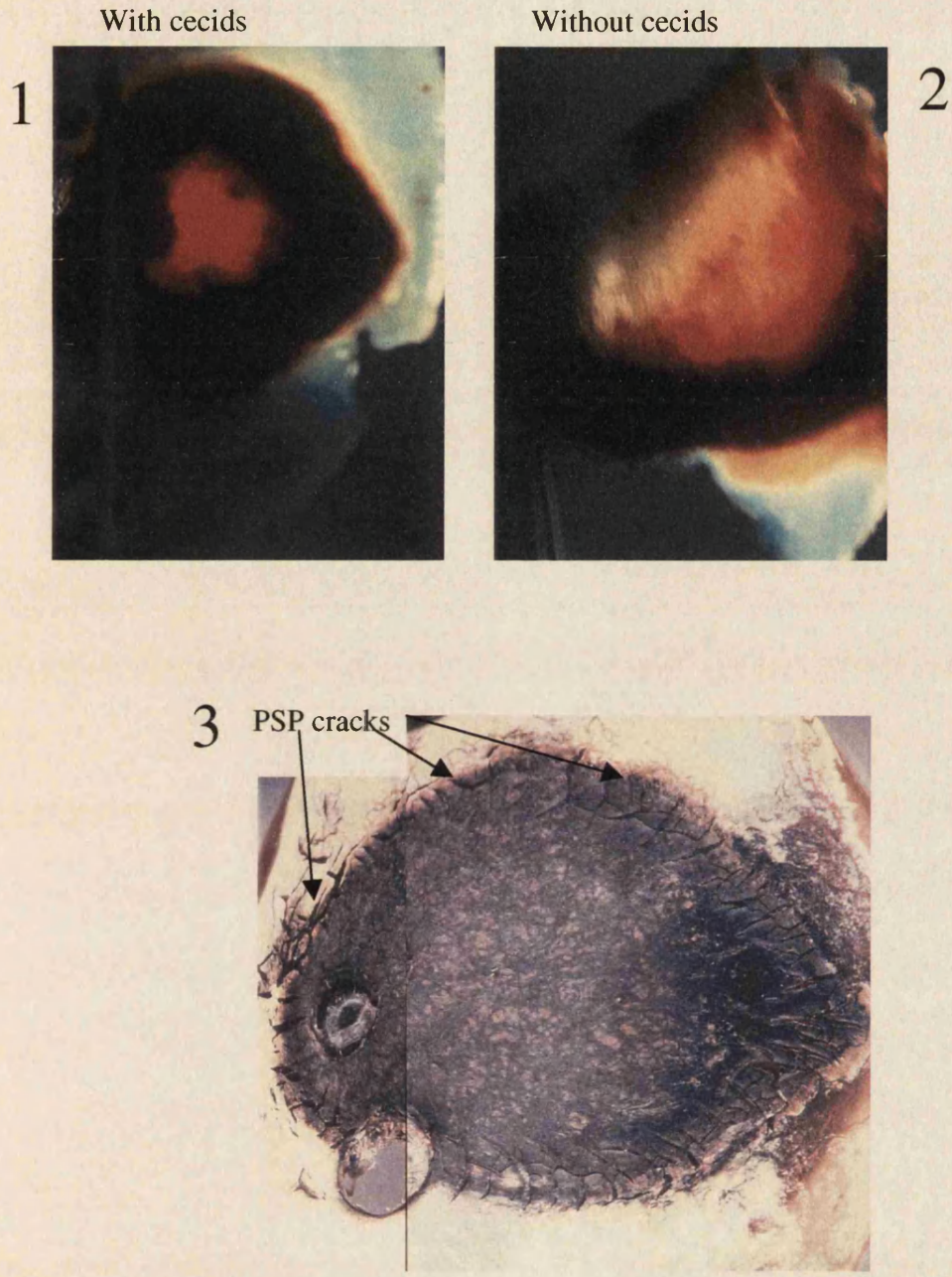


Figure 5.10 (1) + (2) show PSP zones in a "light test" to see thickness. (2) is a thin PSP produced without cecids, (1) a thick PSP produced when combined cultures were treated with cecids. (3) a 6 month old cecid-treated combined culture, desiccated, with large cracks emergent in the PSP zone.

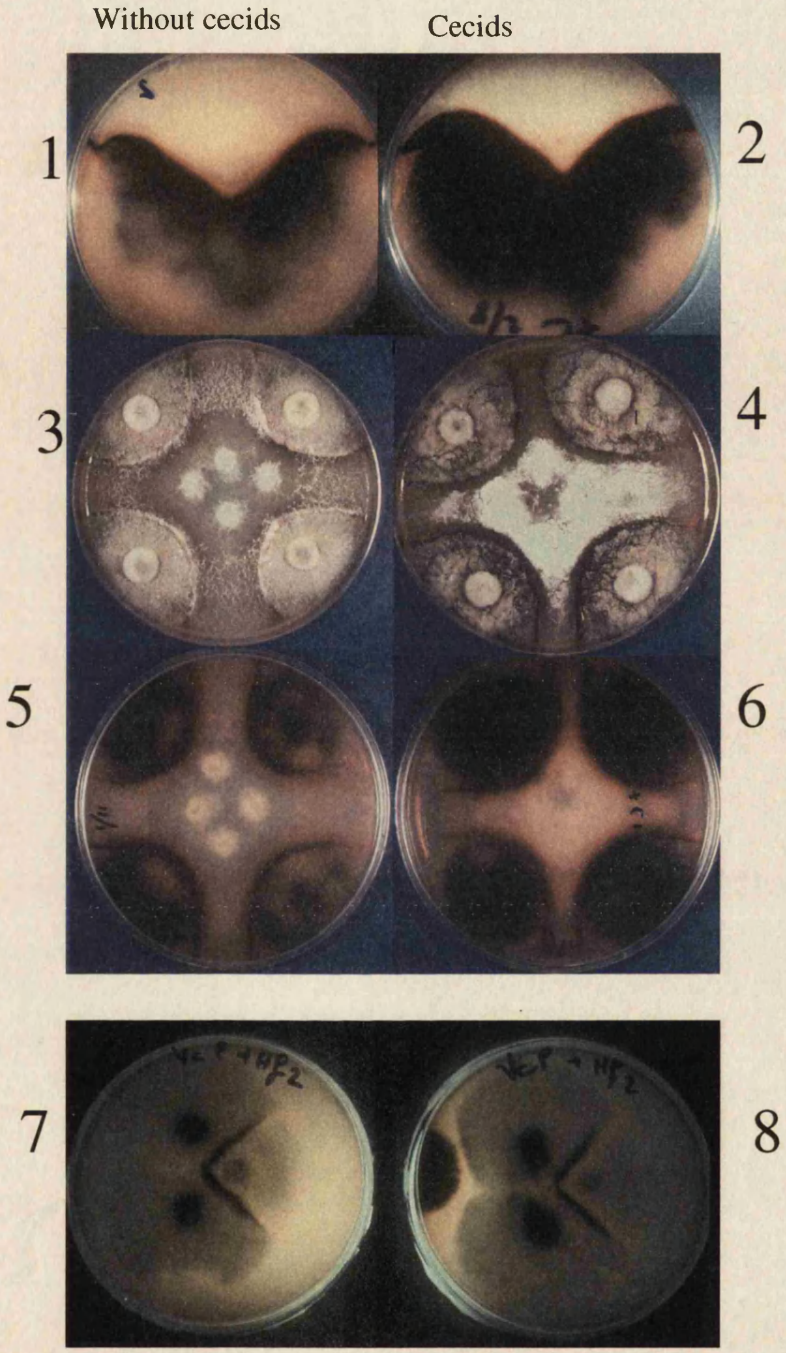


Figure 5.11 Differences between combination culture plates treated with cecids (at right) and without cecids (at left). 1 & 2 show effects of cecids increasing overall *H. fuscum* pigmentation and overall area. 3 & 4 show similar overall effects with *V. Commedens* producing an enhanced aerial mycelium. 5 & 6 show the same culture undersides. 7 & 8 show the difference between two young cultres with the one on the right having some additional interactive mycelia. The *H. fuscum* responds to interaction at it's margin with enhanced pigmentation in the centre. This effect is similar to that seen with cecids.

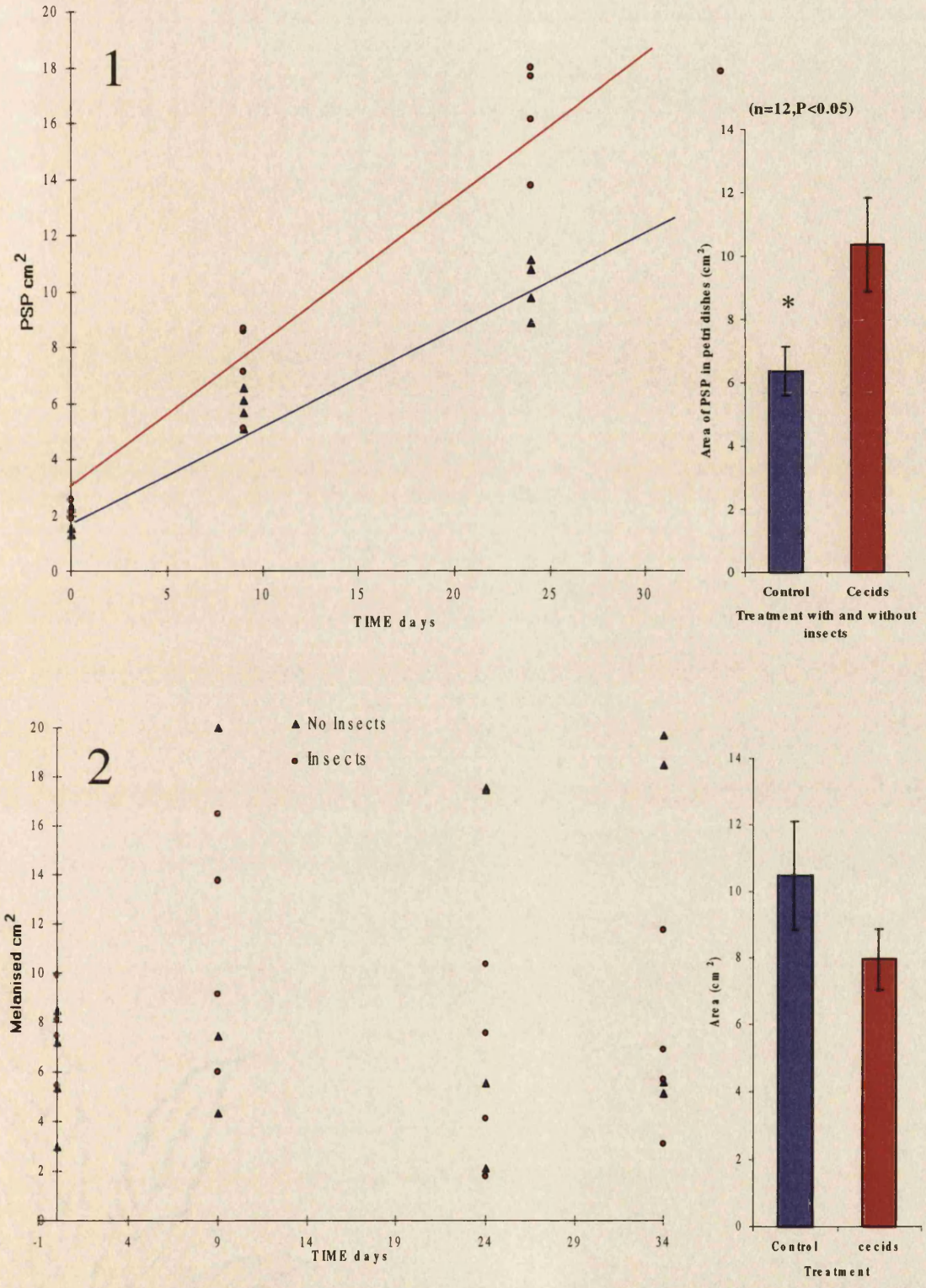


Figure 5.12 Experiment 1: (1) shows significant regressions against time for PSP production with both cecid-treated (red) and controls (blue) b= 0.18 P<0.05; red b= 0.43 P<0.05. The regressions significantly different (T-tested P<0.05) showing that insects enhance PSP production. (2) shows non significant regressions regarding melanised areas over time. *H. fuscum* does not have a significant effect. The insignificant trend for cecids to reduce melanised area (shown in the bar graph) could be an artifact of the conversion rates of melanised area into PSP by cecid-treated interactive cultures.

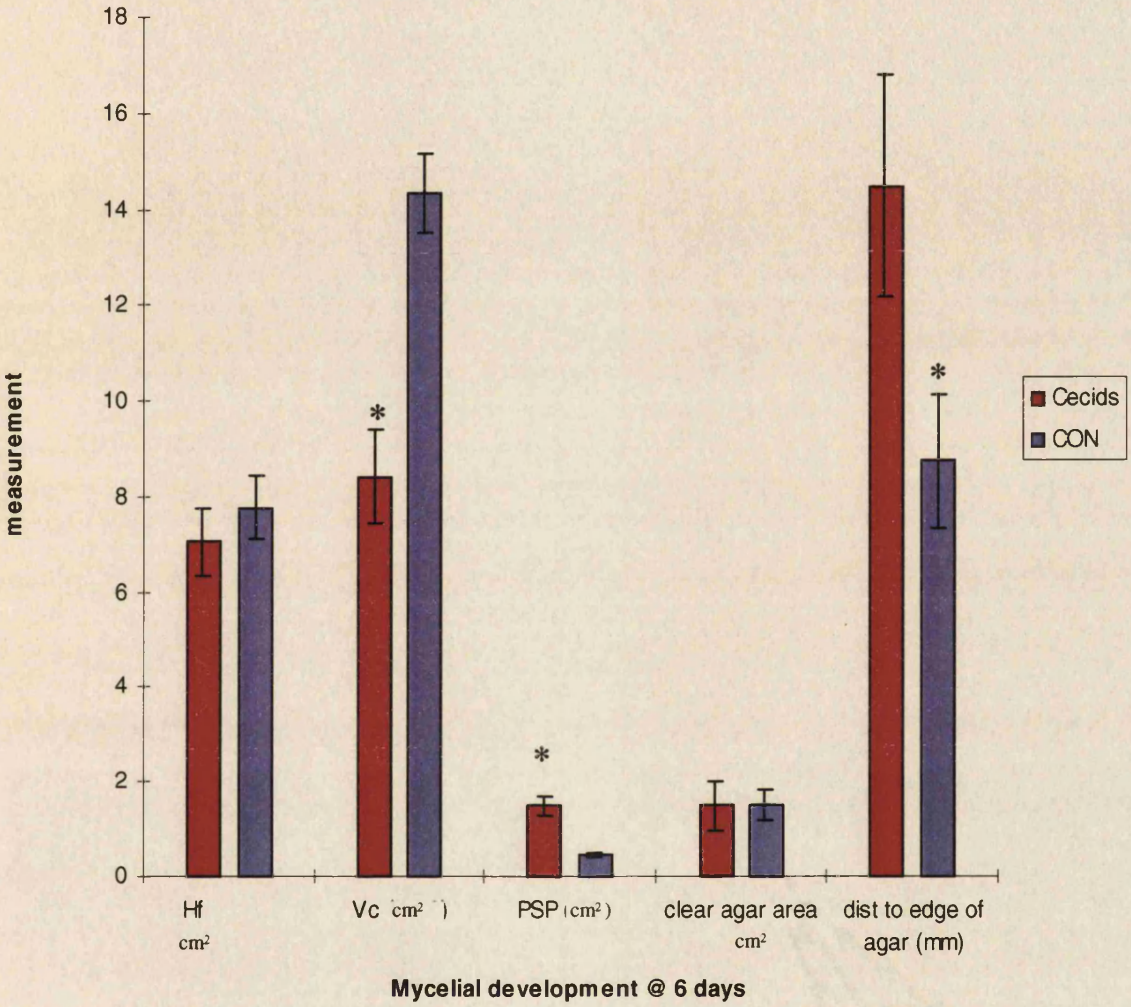


Figure 5.13 Results of experiment 1 pattern 2. A small rep (n=4) combination culture data set 6 days after fungal inoculation and 3 days after cecid introduction. Mann-Whitney non parametric tests found significance ($P<0.05$) between treatments (asterisked) in terms of area of Vc, PSP and distance to the edge of the agar.

Key: Hf = *H. fuscum*, Vc= *V. commedens*, PSP = pseudosclerotial plate, clear agar area = areas of agar with no mycela, dist to edge of agar = distance from mycelial margin, across clear agar, to edge of the Petri plate.

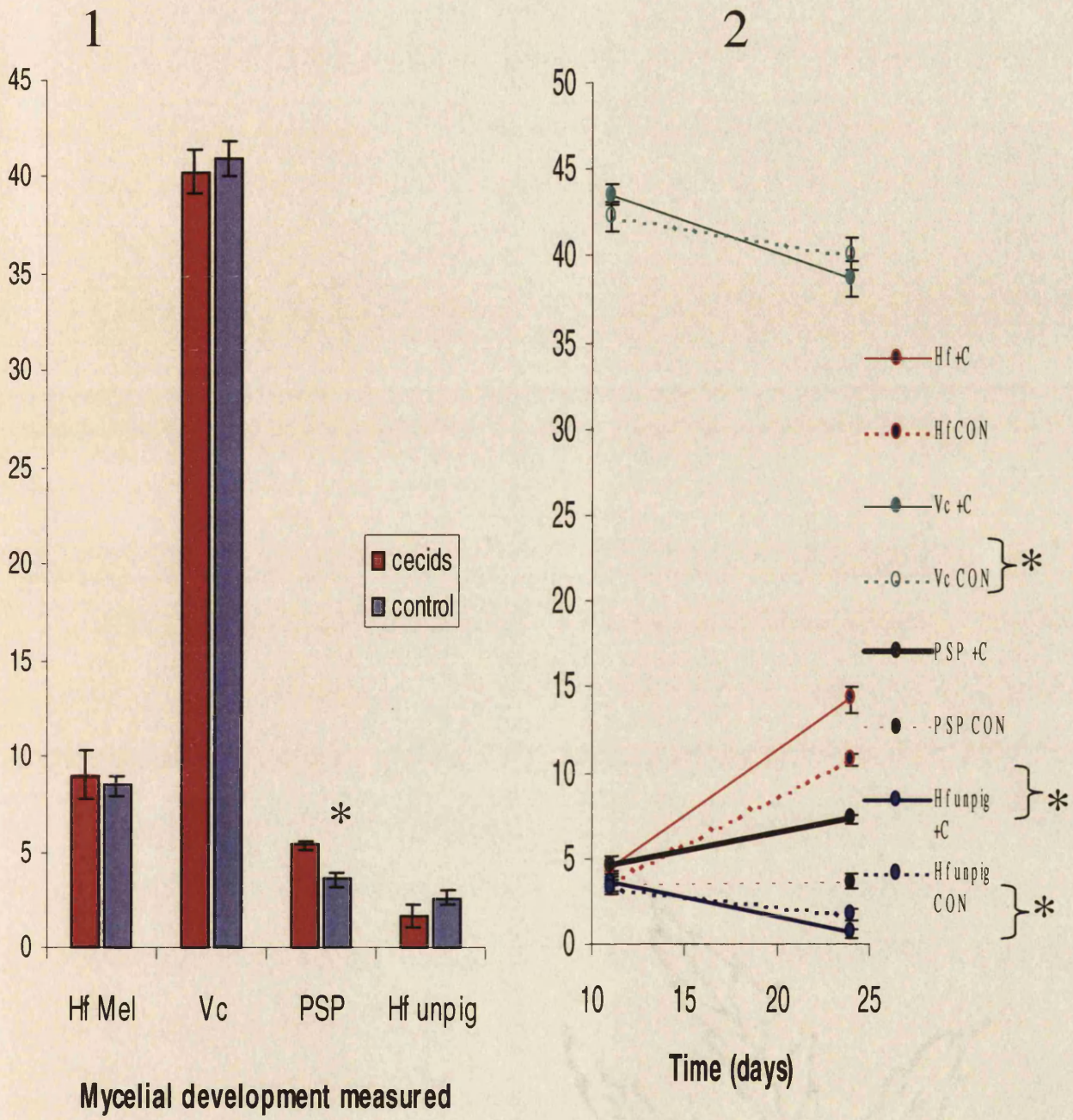


Figure 5.14 Experiment 2 combined cultures measured 11 days after fungal inoculation (4 days after cecid treatments) and 24 days after fungal inoculation (17 days after cecid treatments). (2) shows that at 11 days, no significance between treatments can be detected using the Mann-Whitney non-parametric test (n=9) whereas at 24 days, Mann-Whitney tests show significant difference between PSP area and Hf unpig areas ($P<0.05$). (1) shows amalgamated data from both time periods (n=18). Student t-tests on amalgamated data shows significant difference between PSP areas ($P<0.01$) and Hf unpig areas ($P<0.05$) with cecid treatments. Significant differences between treatments are asterisked.

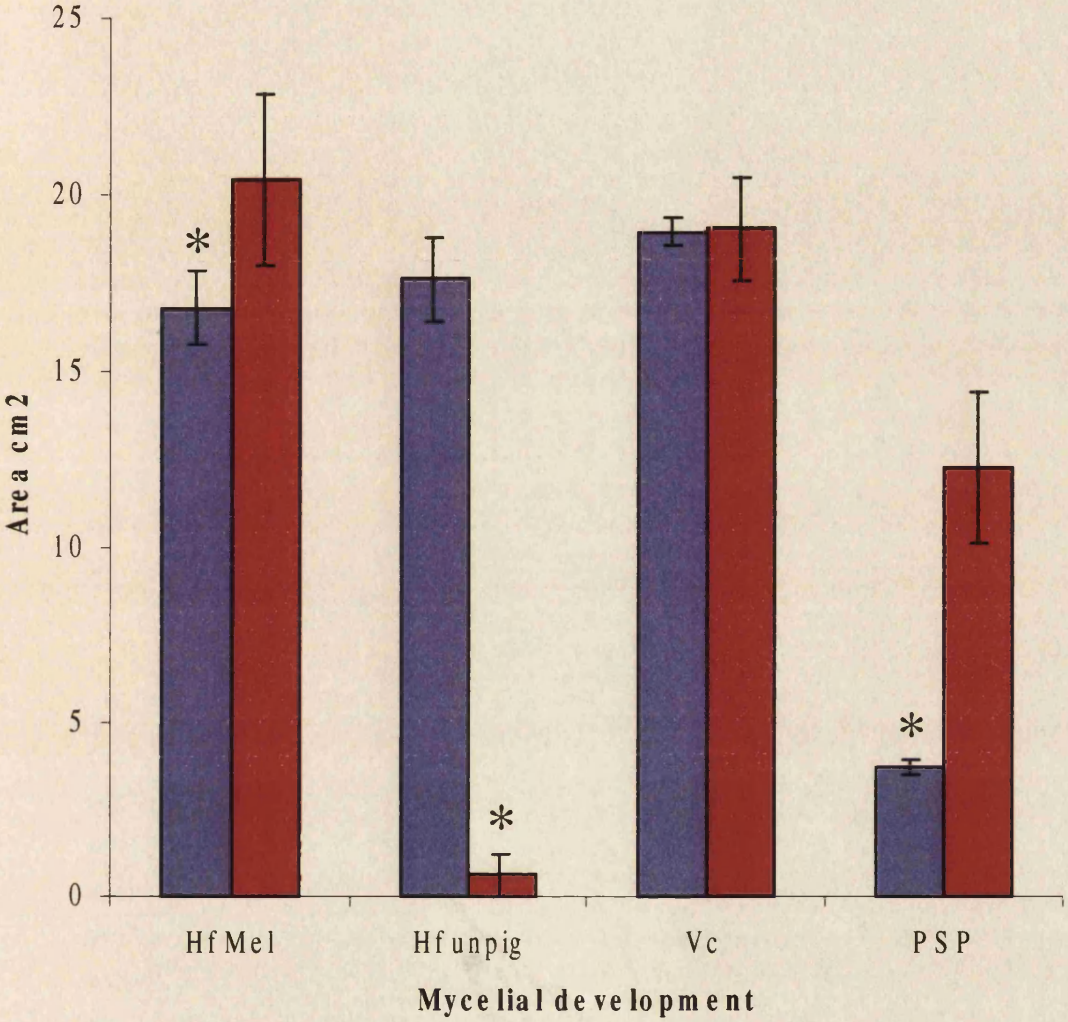


Figure 5.15: Mycelial development for experiment 3 after12 days of cecid influence in treatment of interactive plates in comparison to controls. Controls and treatments used 10 replicates to examine the development of combined culture mycelia with and without treatments of cecid larvae. Using paired t-tests (n=10), significant differences (asterisked) exist between treatments for Hf Mel ($P<0.05$), Hf unpig ($P<0.0001$) and PSP ($P<0.005$). Standard error bars are displayed. **Key**: Hf Mel = Melanised, green-pigmented *H. fuscum*, Hf unpig = young un-melanised mycelium. Vc = Vuilleminia commedens, PSP = pseudosclerotial plate.

5.4.6 Interactive results - the metabolic scale

5.4.6.1 Interactive culture tissue-lysis printing results

5.4.6.1.1 Results of total protein assay

Figure 5. 24 shows results of Coomassie Blue total protein tissue prints. In both treated and control cultures, a reduced amount of protein absorption to the membrane (i.e.less colouration) is visible at the PSP zone. Figure 5.24 shows a subtle mid-PSP protein band and an increase in the amount of membrane-bound protein stained by the dye just on either side of the most recently formed PSP (which is situated at the outer flange extremities, furthest away from central sites of inoculation - where PSP is oldest). A slight overall effect of treatment with cecids can be seen to increase the amount of protein available from inside PSP zones.

5.4.6.1.2 Results of antioxidant assay

Figures 5.25 shows tissue prints for antioxidant activity using DPPH. By comparing the regions of antioxidant activity in *V. commedens* (two regions on the flanges of the most recently formed PSP - away from centre of inoculations) with regions of increased protein binding in figure 5.24, **it can be seen that the most recently formed PSP zone possesses a zone of antioxidant activity on the *V. commedens* side of the interaction boundary which coincides with the band of protein stained with Coomassie Blue. The fact that protein band and antioxidant coincide suggests that this kind of antioxidant is an enzyme.** The effect of cecids was to increase general antioxidant activity in *V. commedens* and inside PSP zones.

5.4.6.1.3 Results of peroxidase activity assay

Peroxidase activity tissue prints are shown in figure 5.26. The effect of cecid treatment seems to be an **overall increase in peroxidase activity inside PSP zones and in *H. fuscum* domains** but a substantial **decrease of peroxidase activity in *V. commedens***. As in total protein prints, it is interesting to note where **peroxidase activity is localised with respect to PSP zones**. Clearly peroxidase forms an important component of the *V. commedens* metabolic repertoire for dealing with oxidative stress. **Cecid trajectories also showed traces of peroxidase activity in their prints, especially in *H. fuscum* domains**.

5.4.6.1.4 Results of catalase activity assay

Catalase activity is shown in figure 5.27 by the presence of oxygen bubbles when leftover developing reagent was poured into Petri dishes until the cultures were completely submerged. This was the clearest way to demonstrate that catalase enzymes were very active in the *H. fuscum* side of PSP zones and that cecid activity seems to reduce this effect. Figure 5.38 shows tissue prints of catalase activity. Tissue prints also demonstrated localised catalase activity near PSP zones that seems reduced by the presence of cecids. Cecids also seem to reduce catalase activity in *H. fuscum* but not in *V. commedens*.

5.4.6.1.5 Results of H_2O_2 localisation assay

The localisation of H_2O_2 production is demonstrated in *V. commedens* zones and on the *V. commedens* side of PSP zones in figure 5.28. PSP zones seem to have less H_2O_2 production, but in cultures with high numbers of cecids, larvae can be seen to have left traces of H_2O_2 activity in their tracks and actual positions at time of

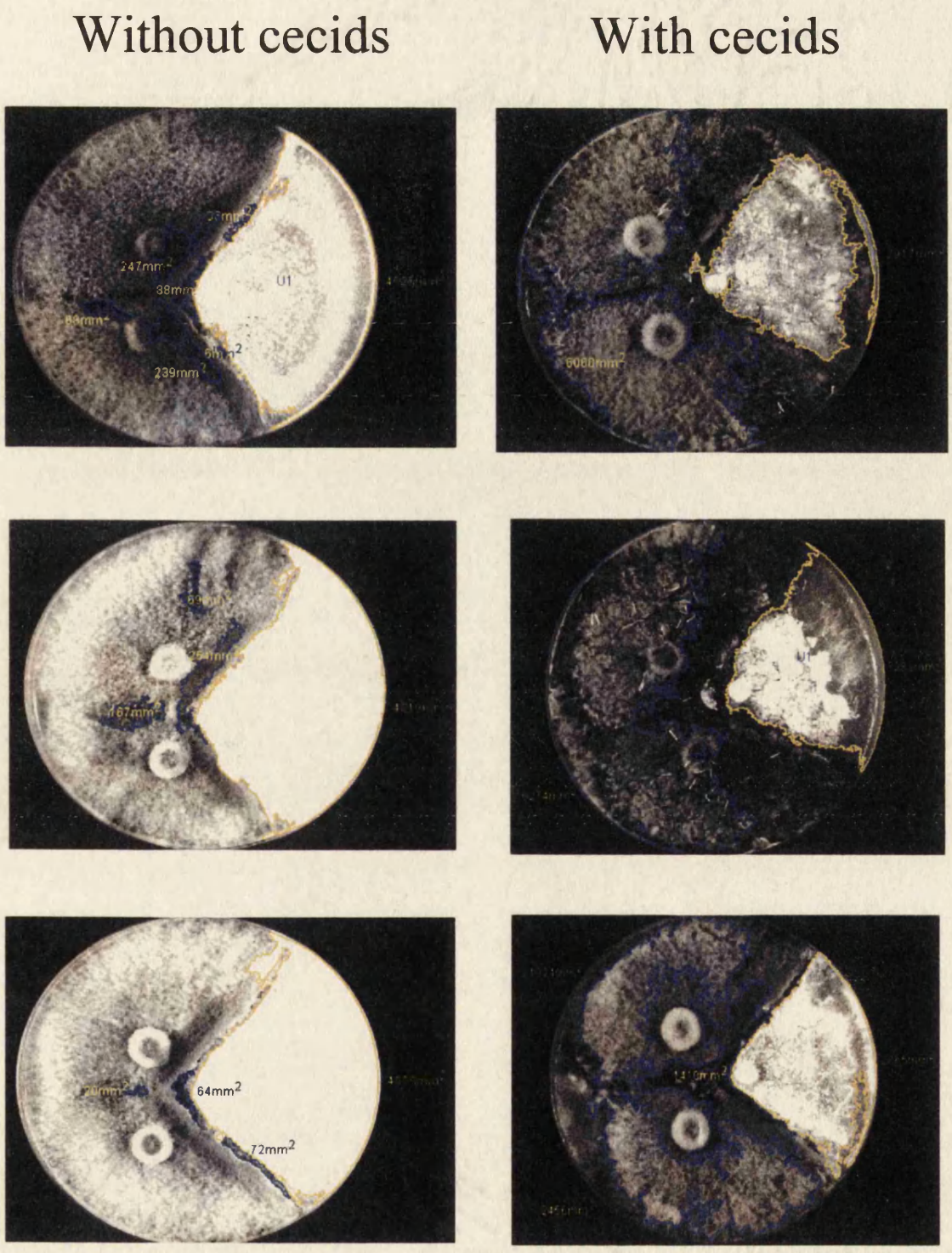


Figure 5.16 Mycelial development contour maps of combination cultures of *Hypoxylon fuscum* and *Vuilleminia commedens* treated with and without cecids.

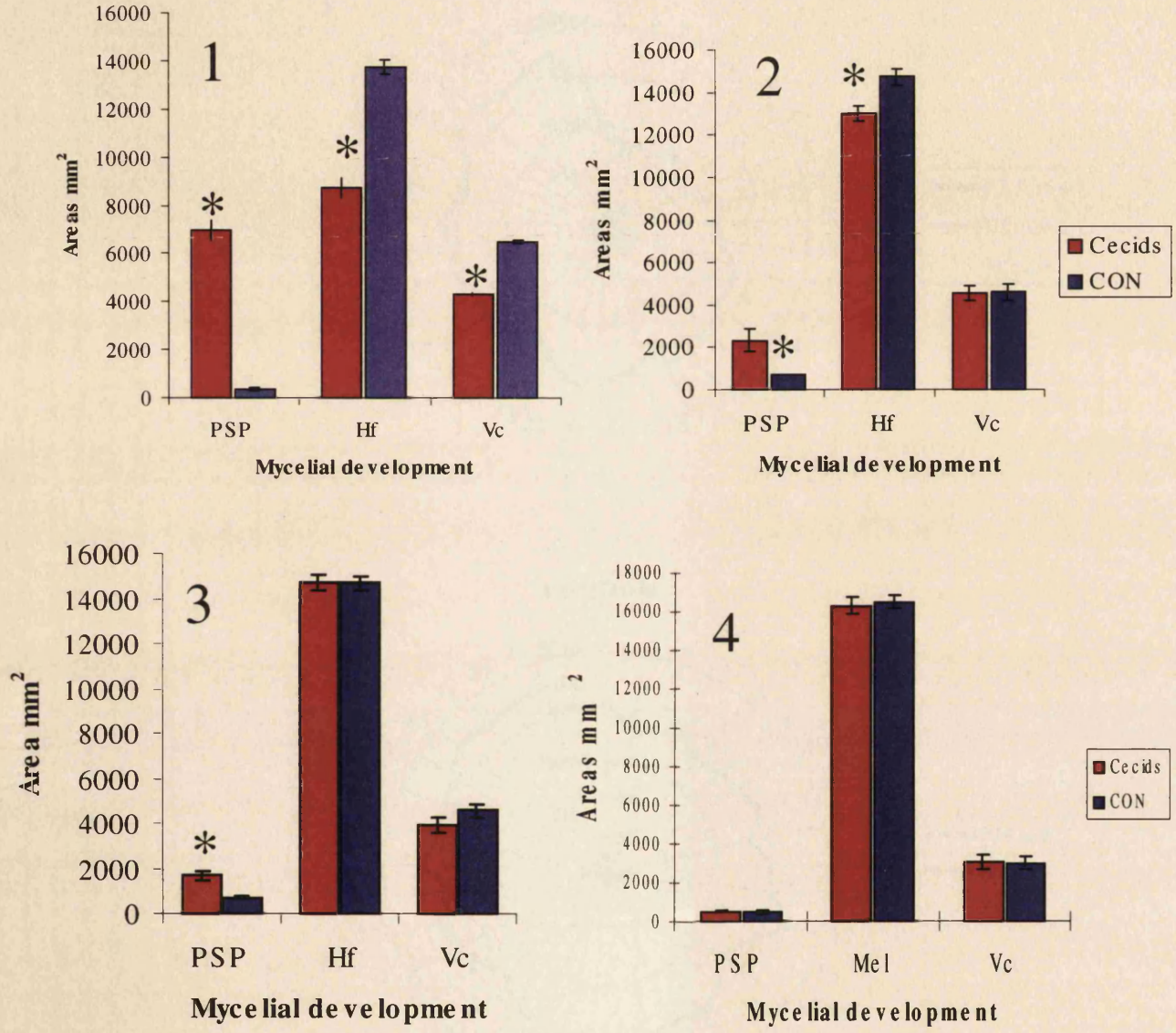


Figure 5.17 Data from combination cultures 1 month after fungal and 3 weeks after cecid inoculation. Data here was derived via image analyses (n=8). Red data sets are cecid-treated interactive cultures, blue are controls without cecid treatment. Asterisks (*) = significantly different results between cecid and control plates ($P<0.05$). (1) shows top orientations of petri-plates, (2) underside view, (3) top view with membrane present for tissue culturing, (4) bottom view with membrane for tissue culturing present.

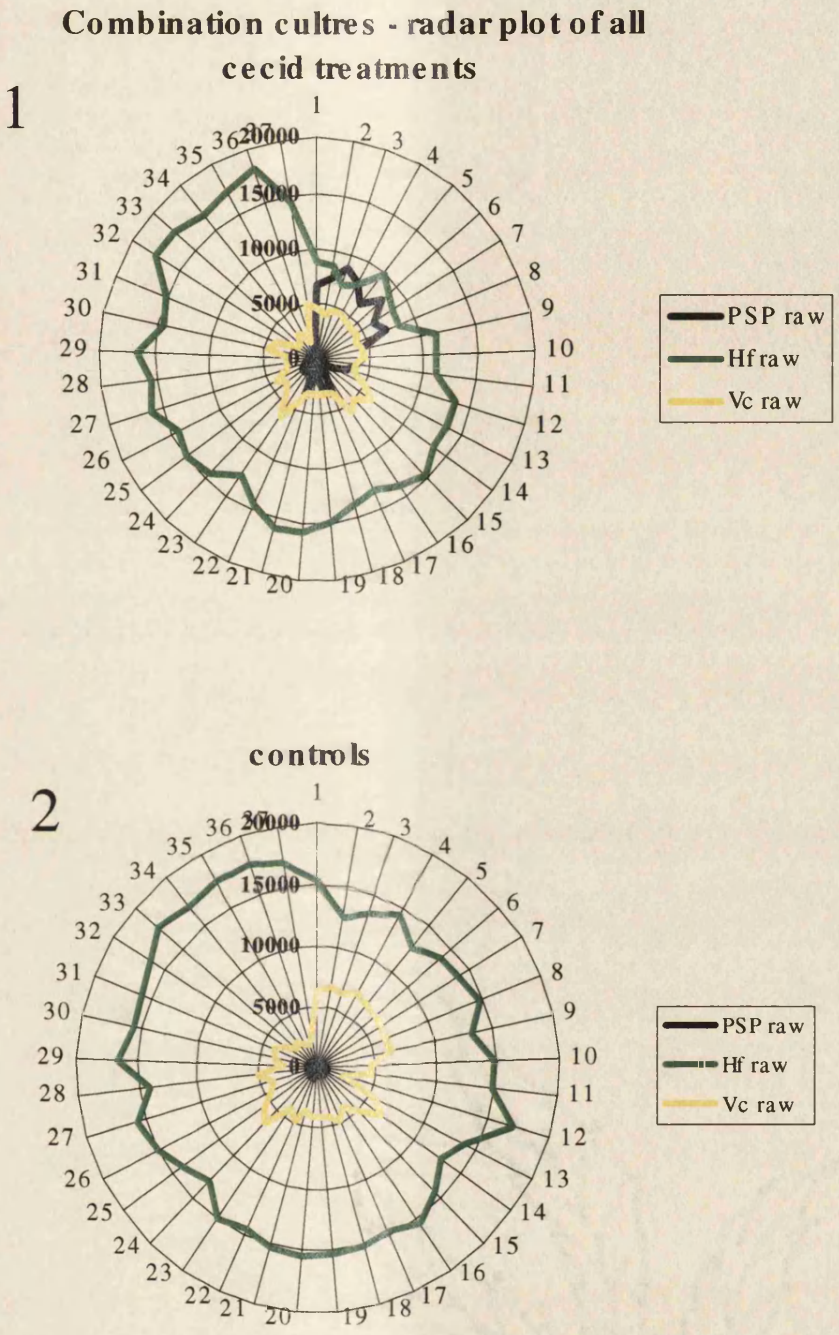


Figure 5.18 Radar plots of amalgamated combination culture data from image analysis. Overlap of lines shows asymmetry, heterogeneity, and nestedness. Smoothness of lines (as in contours around a smooth round hill) indicate a general lack of perturbation in the system.

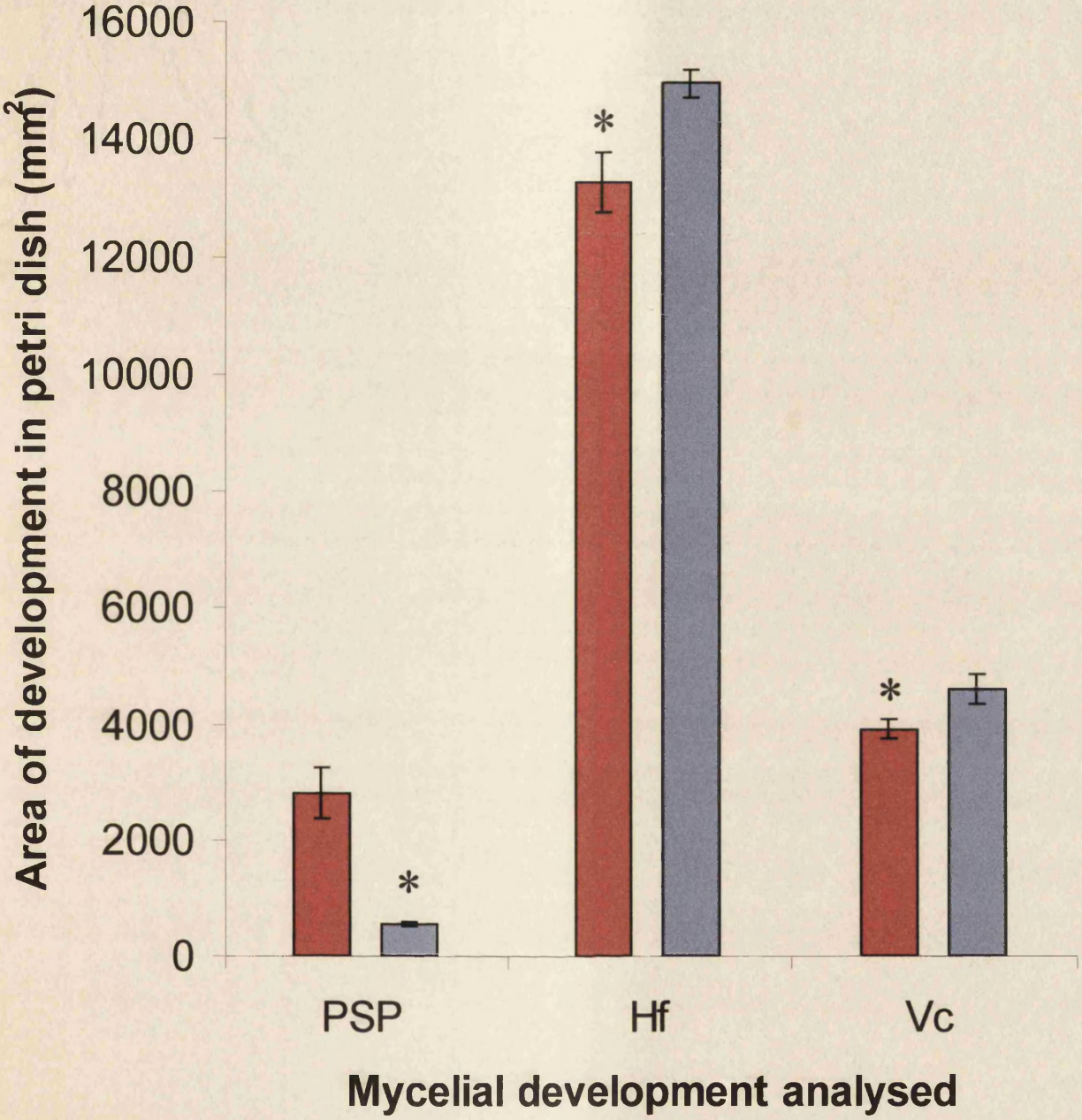


Figure 5.19 Significance of all amalgamated combination culture image analyses giving a large sample size (n=37). Student t-tests performed on data show significance difference P<0.001 (asterisks) for comparisons between treatments of cecids (red) and controls without cecids (blue). **Key**: PSP = pseudosclerotial plate area, Hf = *H. fuscum* area, Vc = *V. commedens* area.

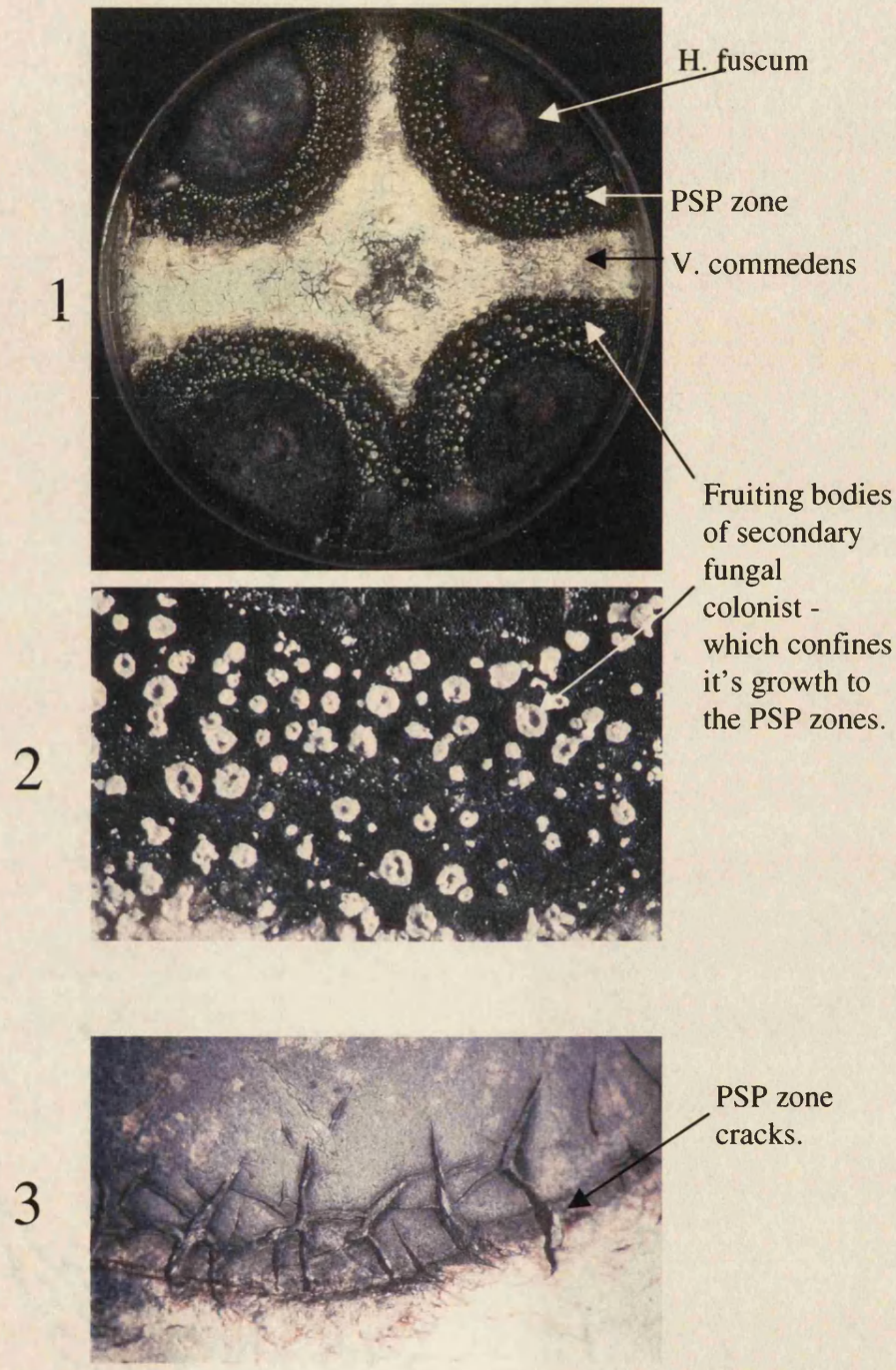


Figure 5.20 Features of the PSP zone's chemistry make it differ from other regions of fungal development. 1 and 2 show how initial chemical conditions of the PSP give rise to a biological potential for secondary colonisation by other fungi. 3 shows how chemical initial conditions can yield a physical potential for enhanced cracking and splitting as a petri-plate's agar looses it's water content.

Figure 5.21 Combination culture with cecids between *H. fuscum* and strain 2 of *Vuilleminia commedens.* The strain 2 isolate is more aggressive, with a more insulated aerial mycelium,and is consequently able to invade *H. fuscum* from over the pseudosclerotial plate (PSP). What is interesting is the effect cecid activity appears to have in reducing this capacity of *V. commedens*-strain 2 from invading the *H. fuscum* domains

V. commedens invasion-fronts are indicated with orange arrows whereas extensive PSP (which cecids enhance) is labelled with white arrows.
The invasion-fronts only continue when aerial mycelium over the PSP zone connects them to the *V. commedens* mycelium.

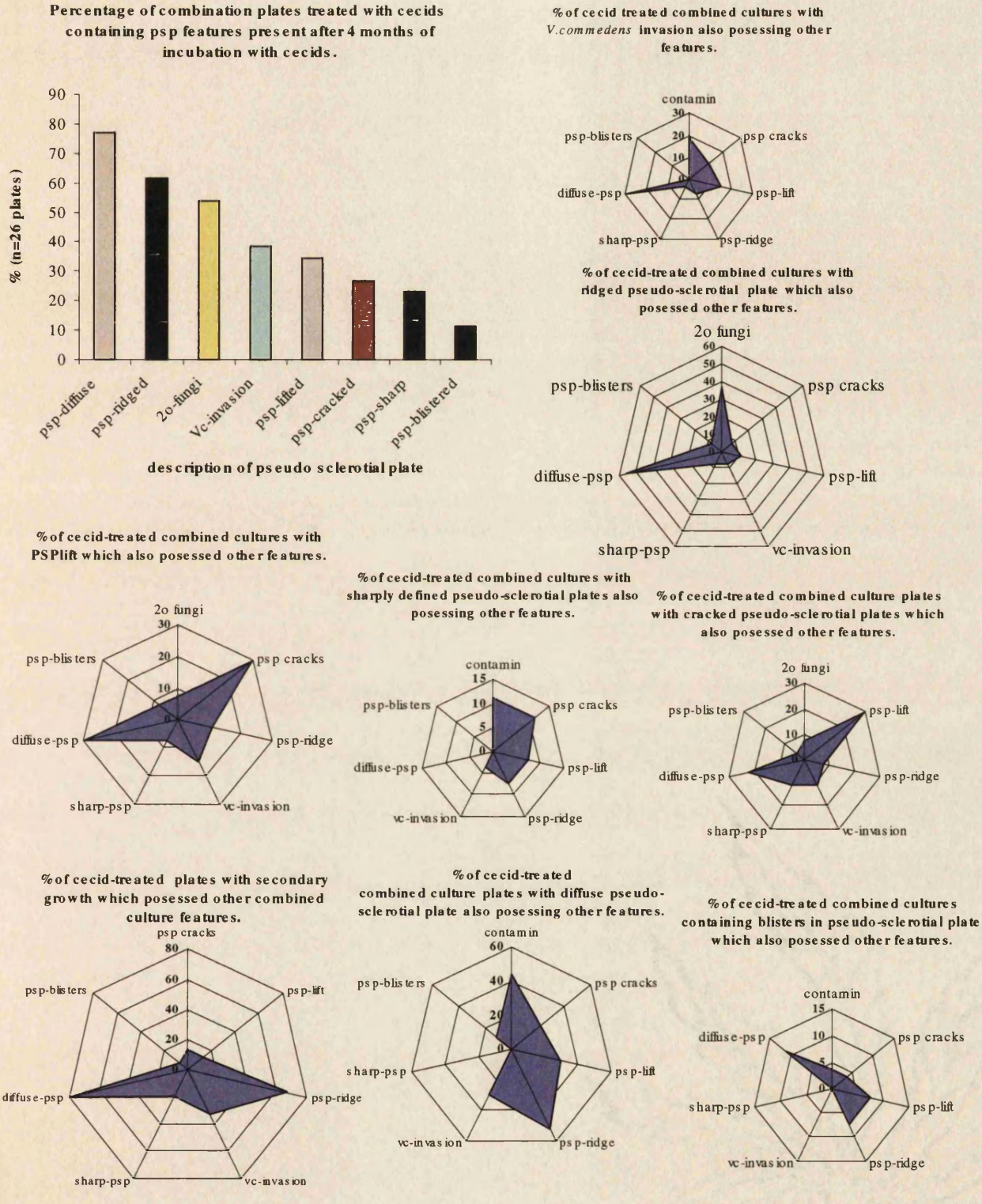
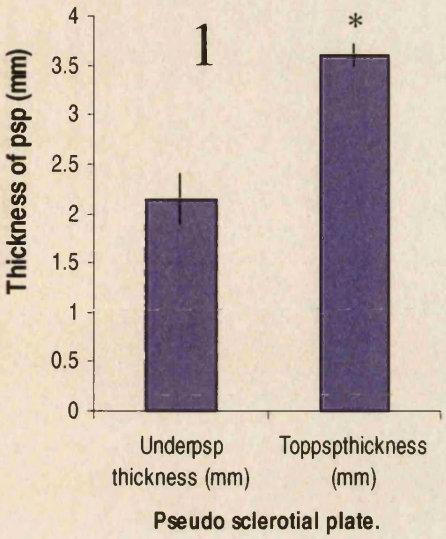
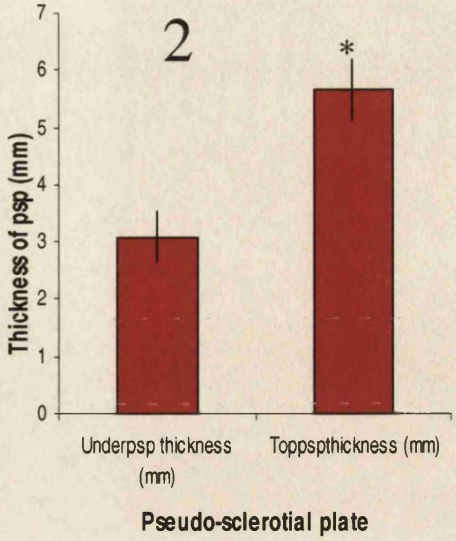


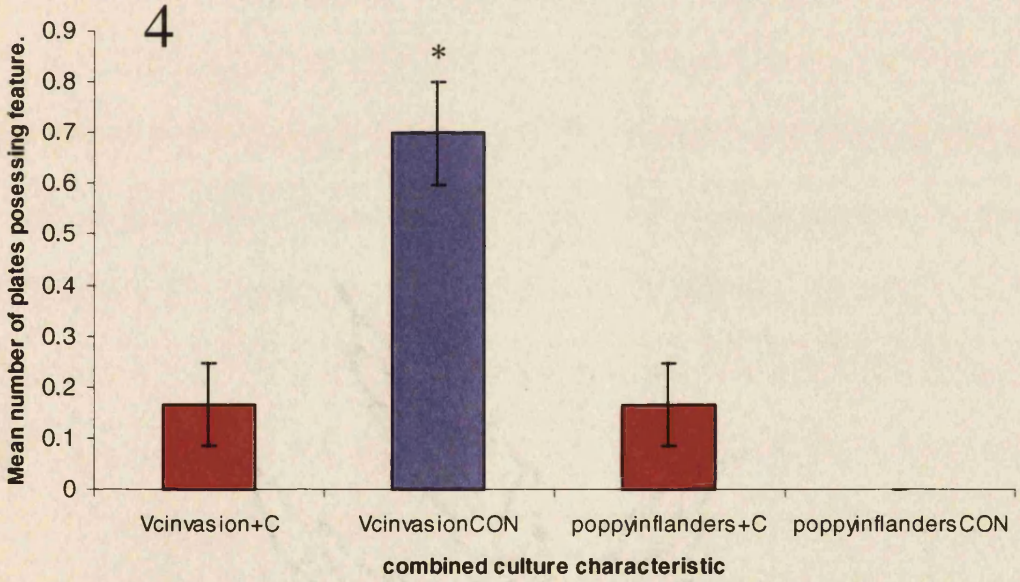
Figure 5.22 Features observed in 26 combined culture Petri plates. Eight radar plots show, for each feature, the amount of relationship with the other features. The statistical table on the following page show significance of these relationships in terms of % overlap and Fishers exact test.





3

Man Whitney	Under	Top
Under	0.63	**0.009**
Top	**0.019**	**0.046**



5

Man whitney	Vc in +C	Vc in CON	Poppy +c
Vc in +C	zero	**0.03**	**0.85**
Vc in CON		zero	**0.027**
Poppy +c			zero

Figure 5.23 (1) and (2) shows comparisons between PSP areas at surface and base of petri-dishes with (2) (n=12, P<0.05) and without cecids (1) (n=12, P<0.05). (3) shows t-test probabilities between variables. (4) shows a comparison of features combined cultures between blue (controls) and red (cecid-treatments) (n=12, P<0.05). "Poppies in Flanders"refers to secondary colonisation particularly of the PSP zone or interactions zones between wood decay fungi (Rayner and Cooke 1984). A table of t-test probabilities for this is shown at (5). Asterisks show significance. Bars displayed are standard error.

Table 5.1 - Fishers' Exact Test probability numbers and % overlap for frequency data of combined culture descriptions.

NB: Fishers exact test was calculated on the raw frequency data and not the % overlap values.

(n=27)

* = $P < 0.05$

MATRIX % OVERLAP GLOBAL (n=27)								
rep	contamination?	PSPcracks?	PSPlift?	PSPRidge?	Vcinvasion?	SharpPSP?	diffusePSP	pspBLISTERS?
contamin	all	7	7	37	19	11	44	4
psp cracks	7	all	30	7	11	11	22	4
psp-lift	7	30	all	11	15	7	30	7
psp-ridge	37	7	11	all	7	7	52	7
vc-invasion	19	11	15	7	all	4	30	0
sharp-psp	11	11	7	7	4	all	0	0
diffuse-psp	44	22	30	55	30	0	all	11
psp-blisters	4	4	7	7	0	0	11	all
Tot	15	8	10	16	9	4	23	3
P no. Fisher's Exact Test (n=27)								
rep	contamination?	PSPcracks?	PSPlift?	PSPRidge?	Vcinvasion?	SharpPSP?	diffusePSP	pspBLISTERS?
contamin	zero	0.087	* 0.007	0.452	1	0.605	0.605	0.569
psp cracks		zero	* 0	* 0.033	1	0.558	0.558	1
psp-lift			zero	* 0.04	0.683	0.613	0.613	0.535
psp-ridge				zero	* 0.011	1	1	1
vc-invasion					zero	1	1	0.529
sharp-psp						zero	1	1
diffuse-psp							zero	1
psp-blisters								zero
Tot								

printing (top left). The effect of cecids seems to have been to increase levels of the H_2O_2 inside PSP zones and *H. fuscum*.

5.4.6.1.6 PSP zone comparisons between tissue-lysis assays

Figure 5.29 shows close ups of control PSP zones with respect to total protein, antioxidant, peroxidase and catalase activity. Older PSP in the centre seems generally less interesting in terms of enzyme and metabolite action. Older PSP seems more stable and less metabolically active than that at flanges. It is here that on the *H. fuscum* side of the more recently formed PSP lies visible evidence of high protein binding, high antioxidant activity and high catalase activity. On the *V. commedens* side of these more recently formed PSP zones, can be seen evidence of high protein binding, distinct lines of low antioxidant activity (which indicates the production of free radicals), high peroxidase activity and a generally high amount of H_20_2 (as shown in figure 5.28). These control tissue print results are summarised in table 5.2 below. The effects of cecids on assay results are summarised below in table 5.3:

Table 5.2 Control tissue prints without cecids showing intrinsic levels of oxidative-stress affecting metabolites on either side of and inside PSP zones which emerge between the ascomycete *H. fuscum* and the basidiomycete *V. commedens*. H = enough reaction in assay to be clearly visible as positive result
L = low visibility of reaction, but with some result - difficult to categorise clearly positive

Tissue print assay	*Hypoxylon fuscum* side of PSP	Inside PSP zone	*Vuilleminia commedens* side of PSP
Total protein	**H**	L	**H**
Antioxidant	**H**	**H**	L
Peroxidase	L	L	**H**
Catalase	**H**	**H**	L
H_20_2	L	L	**H**

Table 5.3 The effects of cecids on results in table 5.2
+ = an increased positivity of assay with cecid treatments
– = a decreased positivity of assay with cecid treatments
0 = no discernable change in positivity of assay with cecid treatments

Tissue print assay	*Hypoxylon fuscum* side of PSP	Inside PSP region	*Vuilleminia commedens* side of PSP
Total protein	0	+	0
Antioxidant	0	+	+
Peroxidase	+	+	–
Catalase	–	–	0
H_20_2	+	+	0

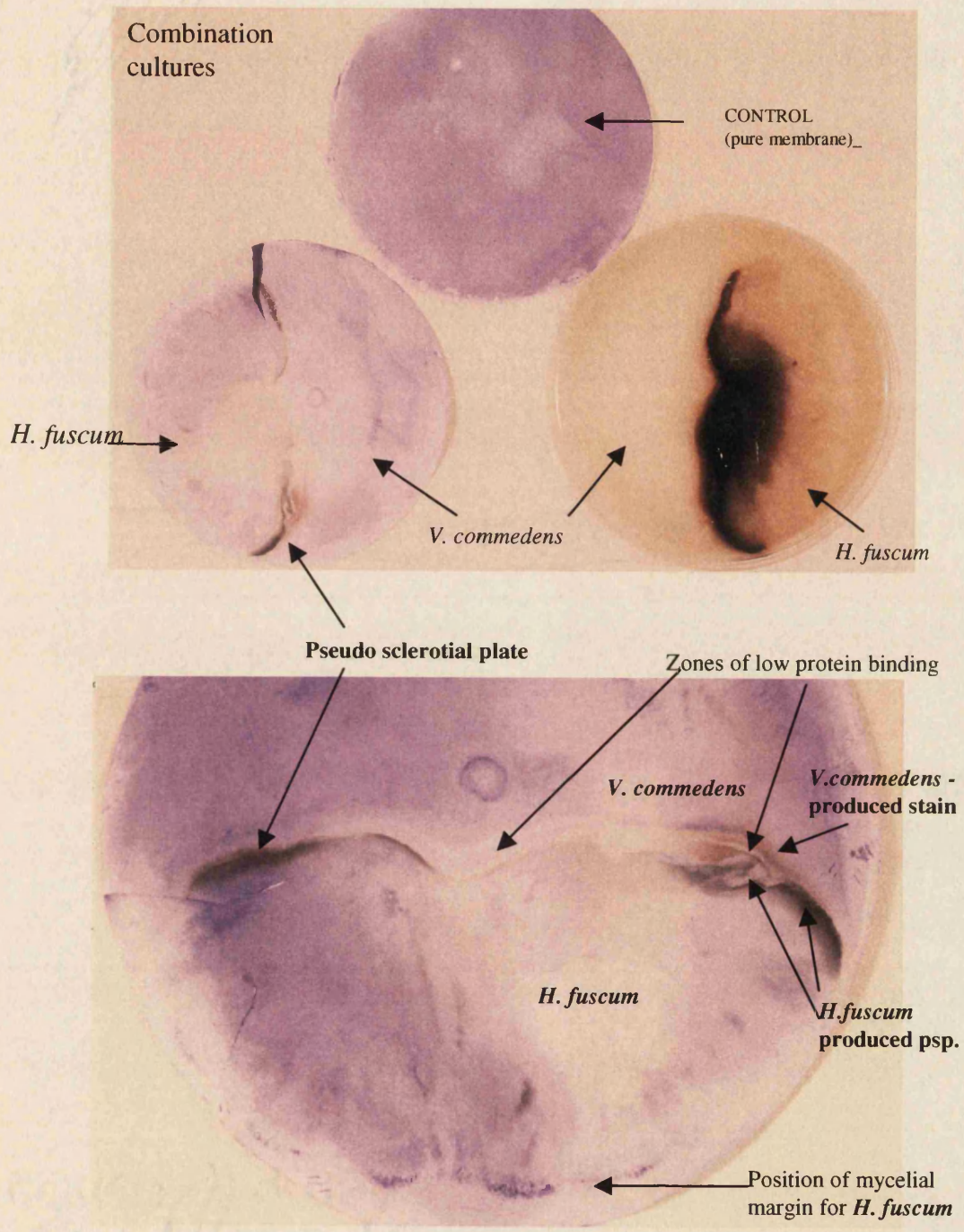


Figure 5.24 Total protein tissue prints made by developing printed nitrocellulose membranes in a coomassie blue assay which stains the majority of bound proteins blue or blue-purple colour.

Combined culture of
H. fuscum and *V.commedens*.

Nitrocellulose membrane ripped off
and sprayed with concentrated solution
of coloured free radical D.P.P.H.

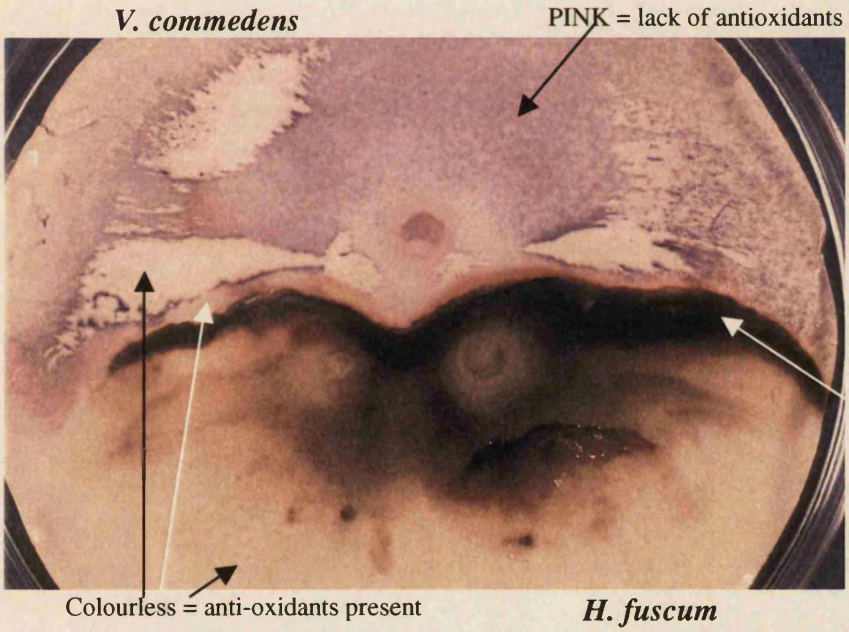


Figure 5.25 Anti-oxidant detecting compound D.P.P.H., used in tissue print assay to determine areas of anti-oxidant activity in combined cultures. The D.P.P.H. solution is a free radical with a pink/purple colour. This colour disappears when the free radicals become sequestered through redox biochemistry by anti-oxidant chemicals or enzymes. In the lower image are the results of the assay, where *H. fuscum* produces a large supply of potent anti-oxidants; some of which filter through the PSP zone to cause clearing on the *V. commedens* side of the boundary. Zones of clearing also occur in *V. commedens* suggesting anti-oxidant activity is limited only to cirtain areas within the mycelium rather than being constitutively expressed throughout.

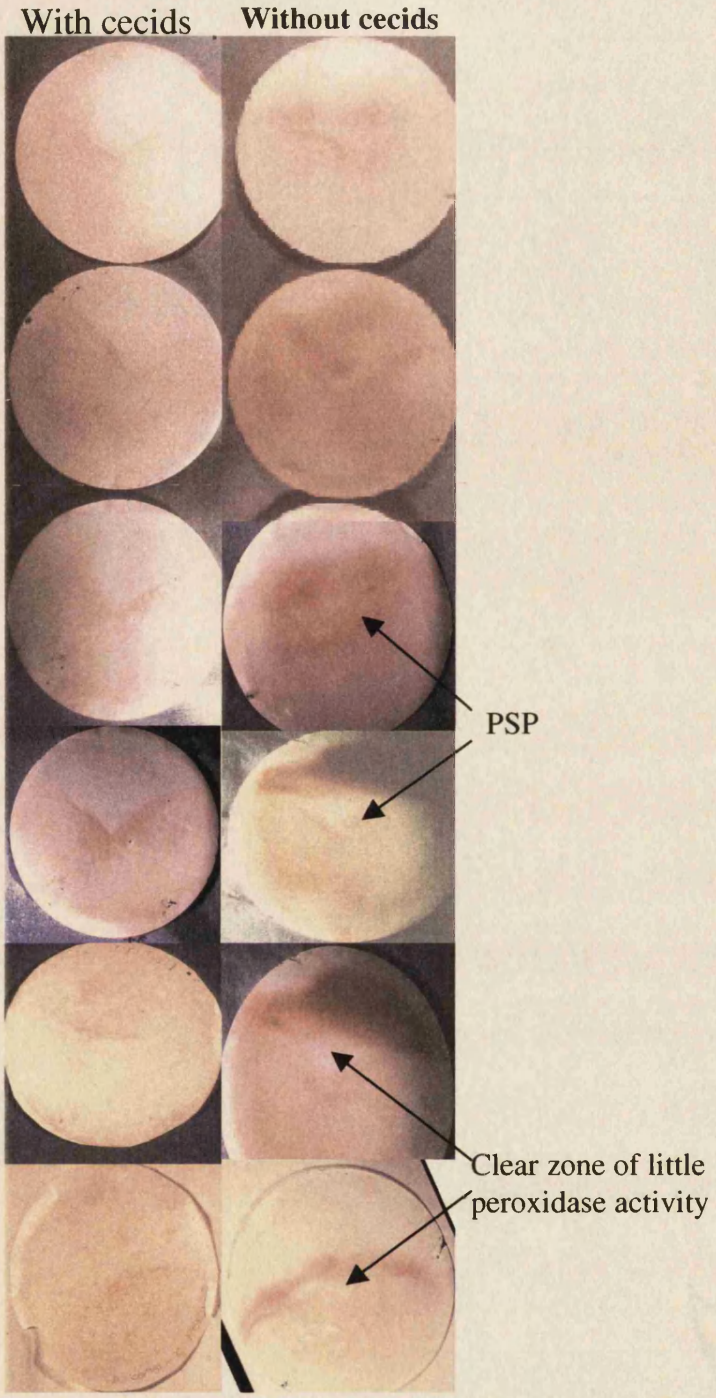


Figure 5. 26 Peroxidase assay tissue prints on combination cultures using nitrocellulose membranes. +ve stain for areas where the enzyme is active result in a brown / blue stain which emerges from the clear developing solution. Cecid treatments run down the left hand side. Each print is orientated with *V. commedens* uppermost. Prints indicate increased *V. commedens* peroxidase activity without cecids. Peroxidase activity follows the PSP zone, but an interesting zone of little activity appears just above the oldest developed PSP (in centre). Cultures with cecids show peroxidase activity near the PSP but it is reduced in area of location and intensity. Some peroxidase activity can be seen in H. fuscum but it remains un-effected by cecids except that they can leave their trajectories as trails of increased peroxidase activity (bottom left).

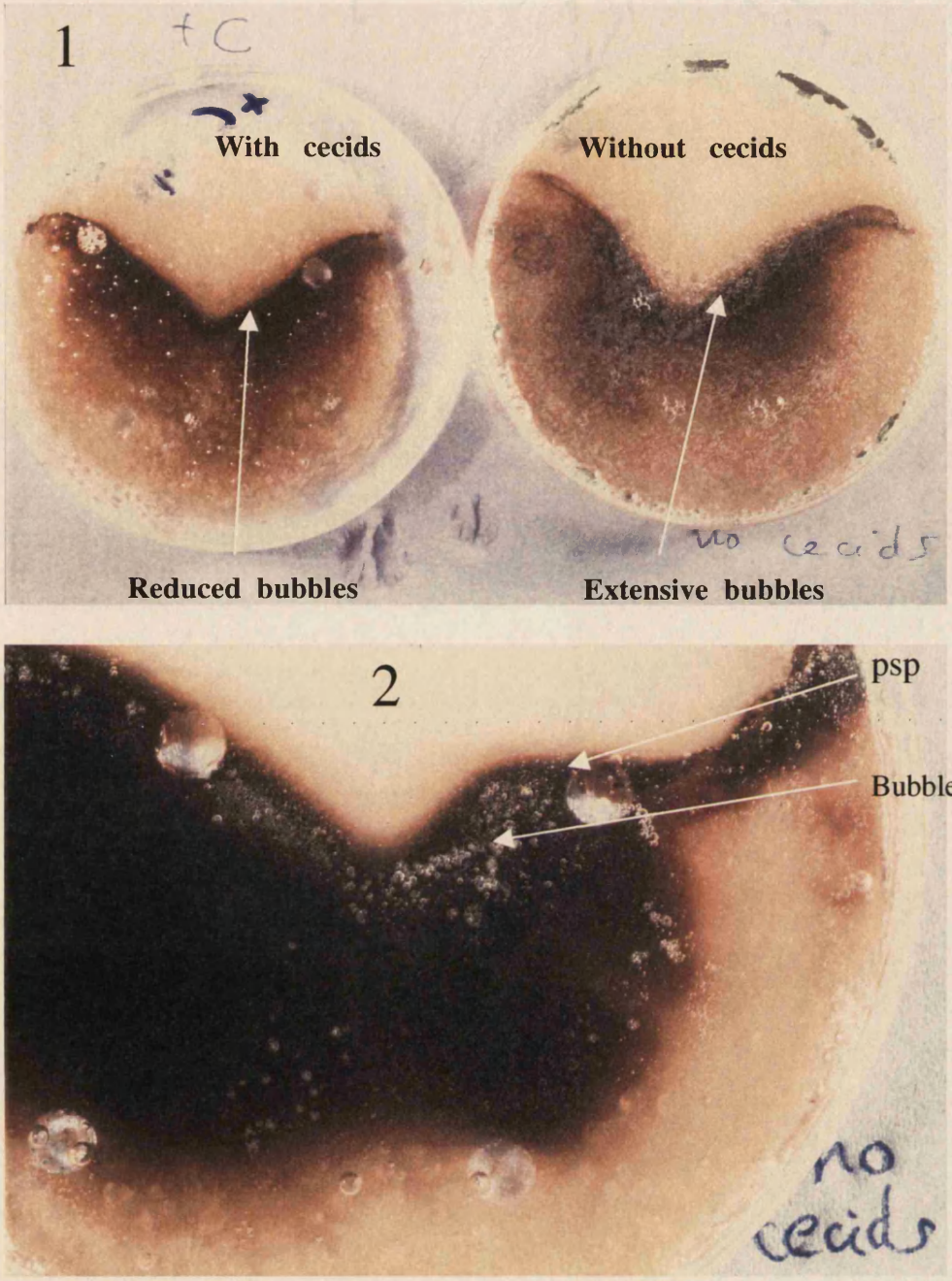


Figure 5.27 Catalase activity detection via bubbles of oxygen released during catalase assay (2). The greatest activity of this enzyme can be seen just to the *H. fuscum* side of the PSP zone. A greater amount of catalase activity is apparent on controls without cecid treatments (1).

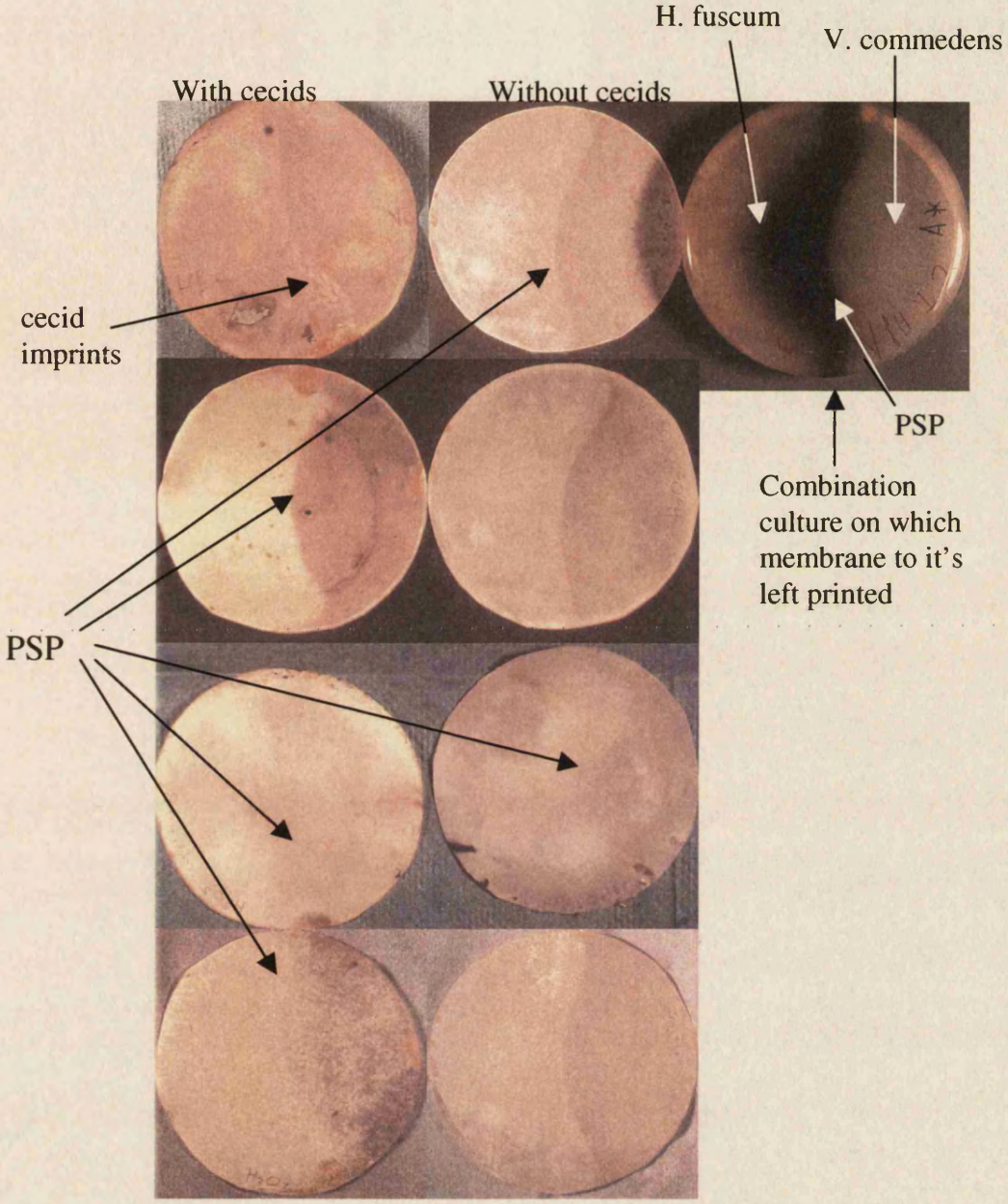


Figure 5.28 H_2O_2 tissue prints of combination cultures made using nitro-cellulose membrane. +ve stain for H2O2 results in the emergence of a dark brown / orange colour change from the original clear reaction solutions. Each membrane has been orientated with the PSP zone running vertically, *H. fuscum* on the left and *V. commedens* on the right. The positions of membranes are such that treatments with cecids are on the left and those without on the right. On the top left print, imprints of cecids can be seen over the clearer *H.fuscum* side of the PSP zone. They appear as dark H_2O_2 -producing cecid-shapes on a lighter background.

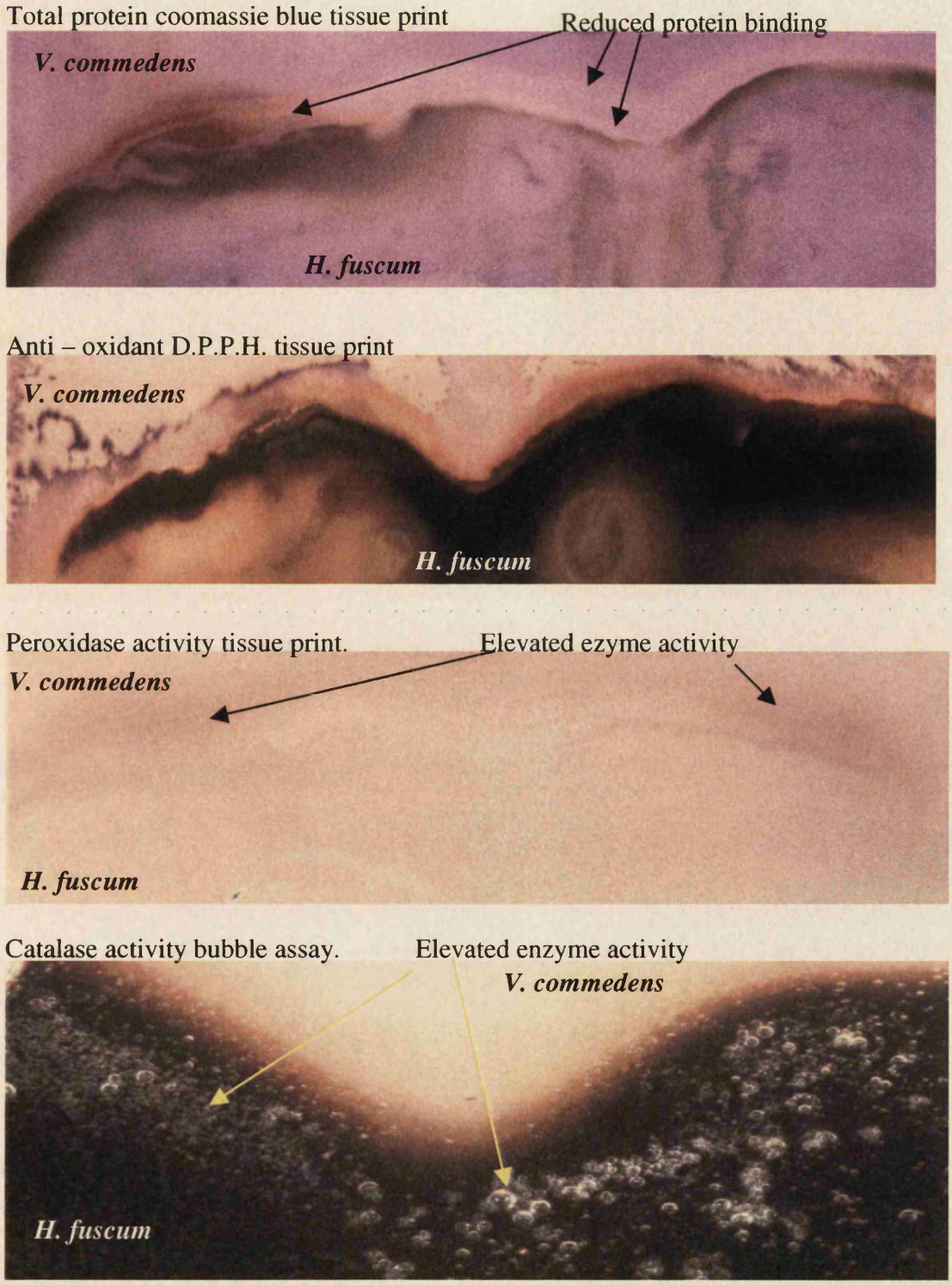


Figure 5.29 PSP zone tissue prints and enzyme activity assays; from top to bottom, Coomassie Blue tissue print for total protein (where +ve=blue), DPPH tissue print for anti-oxidants (+ve=colourless), peroxidase activity tissue print (+ve=brown) and catalase activity bubble assay (+ve= bubble production).

5.4.6.2 Interactive culture HPLC analysis results

Figures 5.30 to 5.34 show HPLC results.

5.3.6.2.1 Comparison of 3 interactive zones

Figure 5.30 shows eluted metabolites from interactive cultures of *H. fuscum* and *V. commedens* and the PSP zone that emerged between them. The retention times of the peaks from different samples can be compared because they are measured at the same wavelength. On the top graph, **interactive *H. fuscum* produces a range of peaks that are absent from *V. commedens* with retention times of 11-12 minutes, 14.5 minutes, and 17 minutes. Also a range of 3 peaks eluting between 15 and 16.5 minutes are reduced to only two peaks in *V. commedens*.** This is also the case on the bottom graph, which adds a third line in a different set of samples. Generally, the compounds that are unique to either *H. fuscum* and *V. commedens* appear together in the metabolite profile for zones of interaction producing PSP. There is one **unique peak in PSP zones that is not present in either of the two fungal profiles and is eluted at 15 minutes**.

5.3.6.2.2 Effect of age on interactive metabolites

The effect of age on the chemical profiles produced from HPLC is shown in figure 5.31. The top two graphs show metabolite profiles monitored at two wavelengths for interactive *H. fuscum* cultures using solvent gradient 1. There is no peak at 15 minutes on either the 210 or 280 nm wavelength spectra. The two graphs below show two triple wavelength spectra: one for young interactive *H. fuscum* (3 weeks old) and one for old interactive *H. fuscum* (7 weeks old). **Several changes have taken place in the old *H. fuscum* culture. For example, between 10 and 12 minutes a range of new peaks has appeared at 350 and 215 nm wavelength. Similarly new peaks have emerged between 16 and 18 minutes in the profiles monitored at all three wavelengths. Conversely there are also two losses in the older culture - first a peak at 20 minutes is lost from the 215 nm wavelength spectra, and another peak at 24 minutes is lost from the 215 nm spectra.**

5.3.6.2.3 Effect of distance from the PSP zone on metabolite profiles

Figure 5.32 shows orientation of HPLC results for the PSP zones sandwiched between *H. fuscum* and *V. commedens* as they would be in culture with distance away from the PSP zone increasing towards the upper and lower margins of the page. Despite the differences between solvent gradient systems, meaning that only shapes of chemical profiles can be compared, general trends can be seen in shifts in the polarity spectrum of eluted compounds. General **trends show a shift to the right towards non-polar molecules in PSP zones.** This statement can be made with confidence on the basis of using the **triple peak, which occurs in all *H. fuscum* and PSP profiles** as a reference with which to compare the retention times of all other peaks. As samples were removed from regions of the plate that were closer to the PSP zone, the triple peak gets further to the left in respect to the overall profile. The same principle was used looking at *V. commedens* using the tallest peak produced as a reference point to observe that, as with *H. fuscum*, as the PSP zone is approached, more metabolite peaks occur to the right.

5.3.6.3.4 Detailed comparison of three interactive zones

Figure 5.33 shows 3 triple wavelength spectra for a finer scaled comparison of the metabolite profiles of interactive mycelia and the PSP zone. In this figure all but the black and white spectra (4) can be compared using peak retention times. All samples were taken from 8 week old interactive plates. **The metabolite profile of the PSP zone can be seen to be made of peaks present in the *H. fuscum* profile at 10, 12, 14, 18, 22 and 24.5 minutes**. From the other side, the ***V. commedens* metabolite profile contributes peaks at 24 minutes**. Furthermore, the **PSP zone profile also has two unique peaks at 20 minutes and 26 minutes**. These latter peaks are very non-polar molecules. These detailed changes agree with general trends of the previous figures and also the black and white lined HPLC spectrum of figure 5.33 (4), which shows a comparison between interactive *H. fuscum* and PSP zone at 250 nm wavelength. Thus there is a general trend with **a shift of peaks generally to the right (non-polar) with the hatched line for PSP zone** metabolites.

5.3.6.3.5 Effects of cecids on 3 interactive metabolic profiles

Figure 5.34 shows the effects of cecids on the HPLC metabolite profiles of the 3 interactive domains - *V. commedens*, PSP and *H. fuscum*, as described below.

5.3.6.3.5.1 Effect of cecids on V. commedens metabolite profile

New peaks emerge in *V. commedens* cultures with cecids. These peaks elute at 12.5 and 29 minutes. Conversely, peaks are lost from the *V. commedens* metabolic profile at 9. 5, 11, 16.7 and 24 minutes when cecids are present.

5.3.6.3.5.2 Effect of cecids on PSP zone metabolite profile

Considering PSP zones, **new peaks emerge at 19 and 21 minutes** in cecid treated cultures. Conversely, **losses of peaks from PSP profiles occur at 7, 9.5, 10.5, 12.5, 18 and 26 minutes**. In addition there is a repeat finding of a **unique PSP zone metabolite** that is not found in either of the interactive fungi, nor does it appear to be induced by cecid activity. This **PSP-unique metabolite elutes from solvent gradient system 2 at 15 minutes.**

5.3.6.3.5.3 Effects of cecids on H. fuscum metabolite profile

Considering *H. fuscum*, **new emergent peaks occur at 7.5, 9.5, 14.5, 24.5 and 27 minutes** and **one peak is lost at 16 minutes** with cecid cultures.

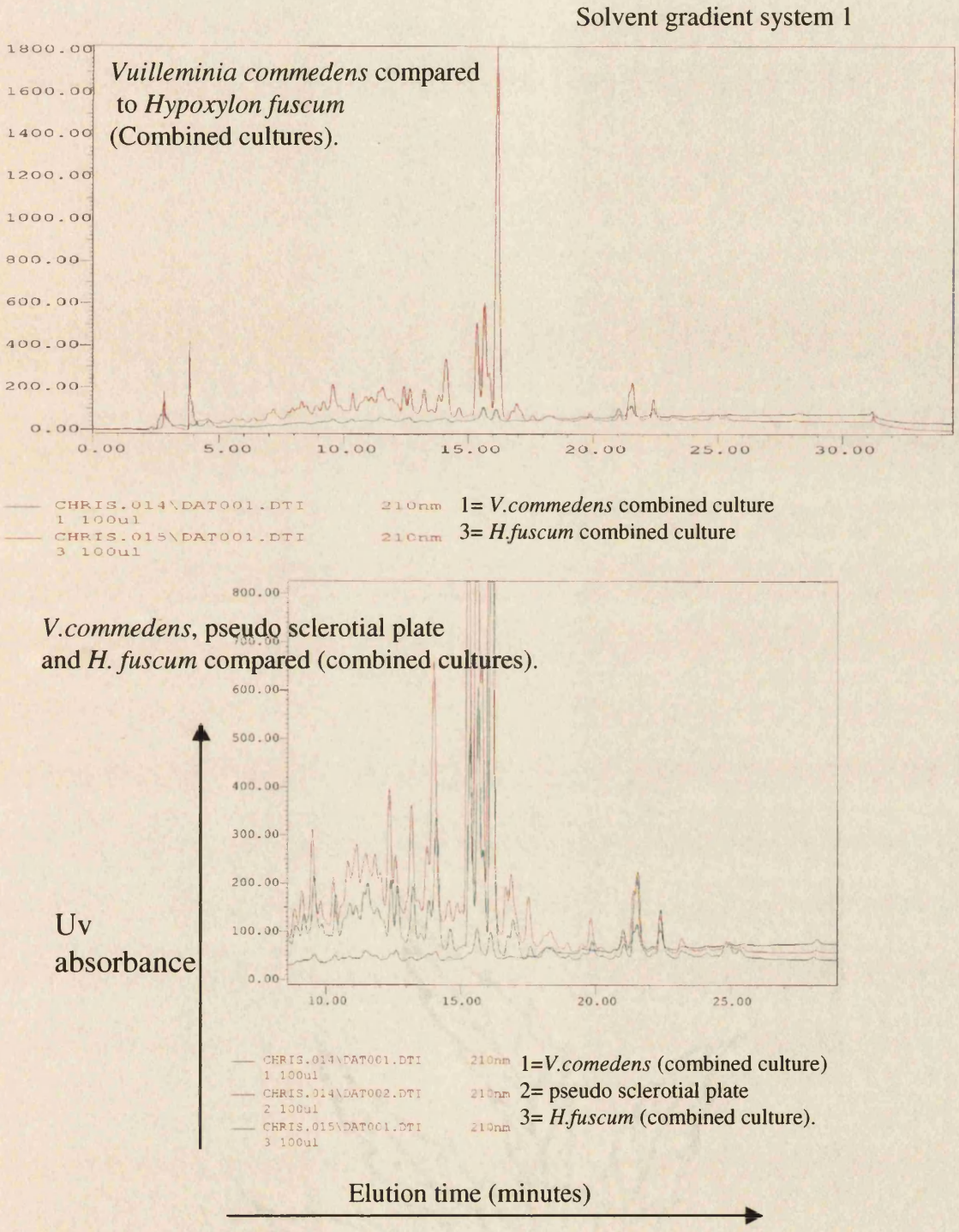


Figure 5.30 H.P.L.C. metabolite profiles compared between different fungi at detection wavelength 210 nm. (2 runs with 5 samples.)

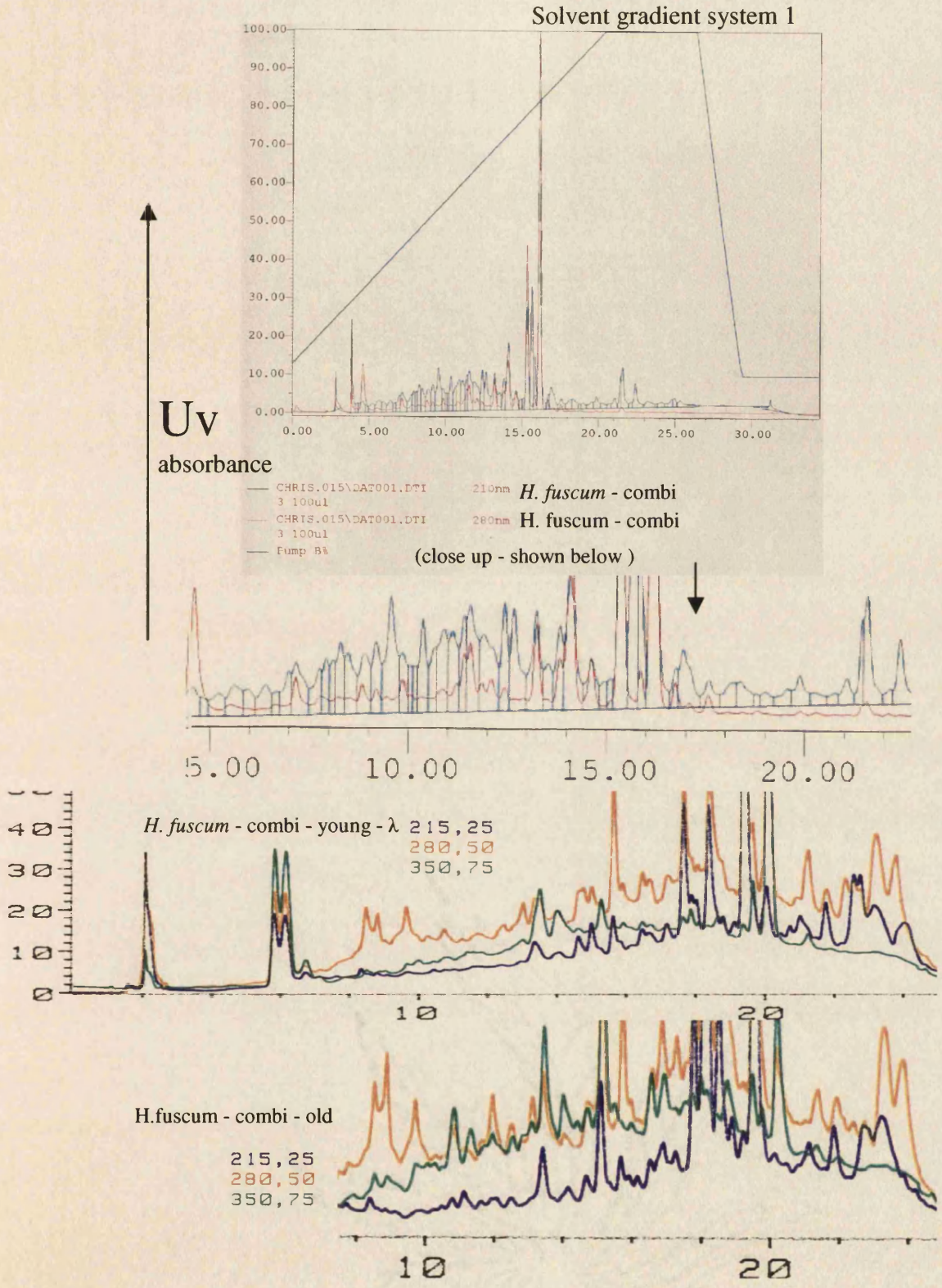


Figure 5. 31 Effect of age; H.P.L.C. metabolite comparisons (2 runs with 3 samples) for combined culture *H. fuscum* at different detection wavelengths.

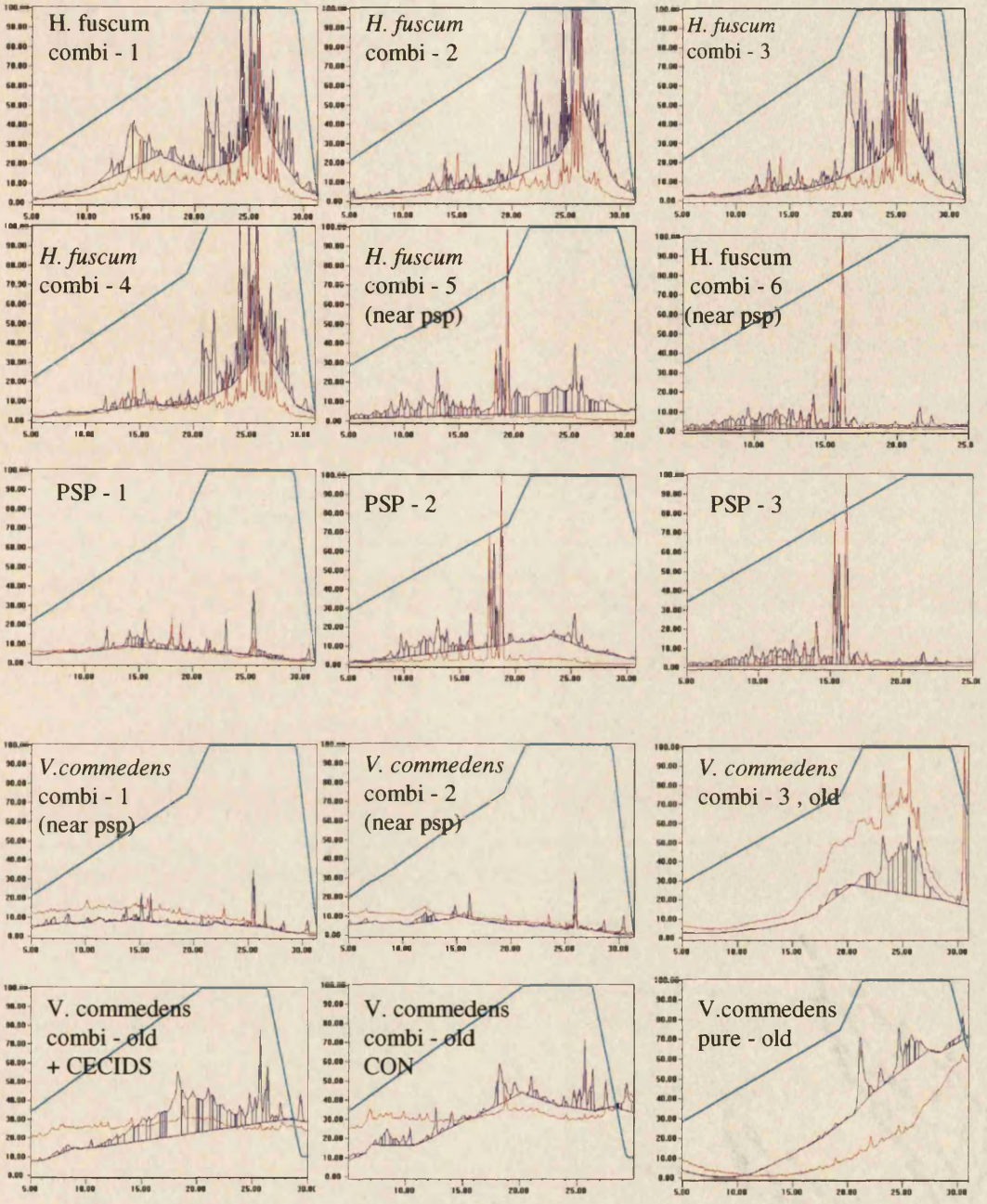


Figure 5.32 HPLC metabolite comparisons (1 run with 15 samples) from combination cultures aligned so as to see general trends in polarity of metabolites in gradation from left to right, from un-polar to highly polar. Top 6 chromatographs are from *H. fuscum*, middle three are from PSP zone, and bottom 6 are from *V. commedens*.

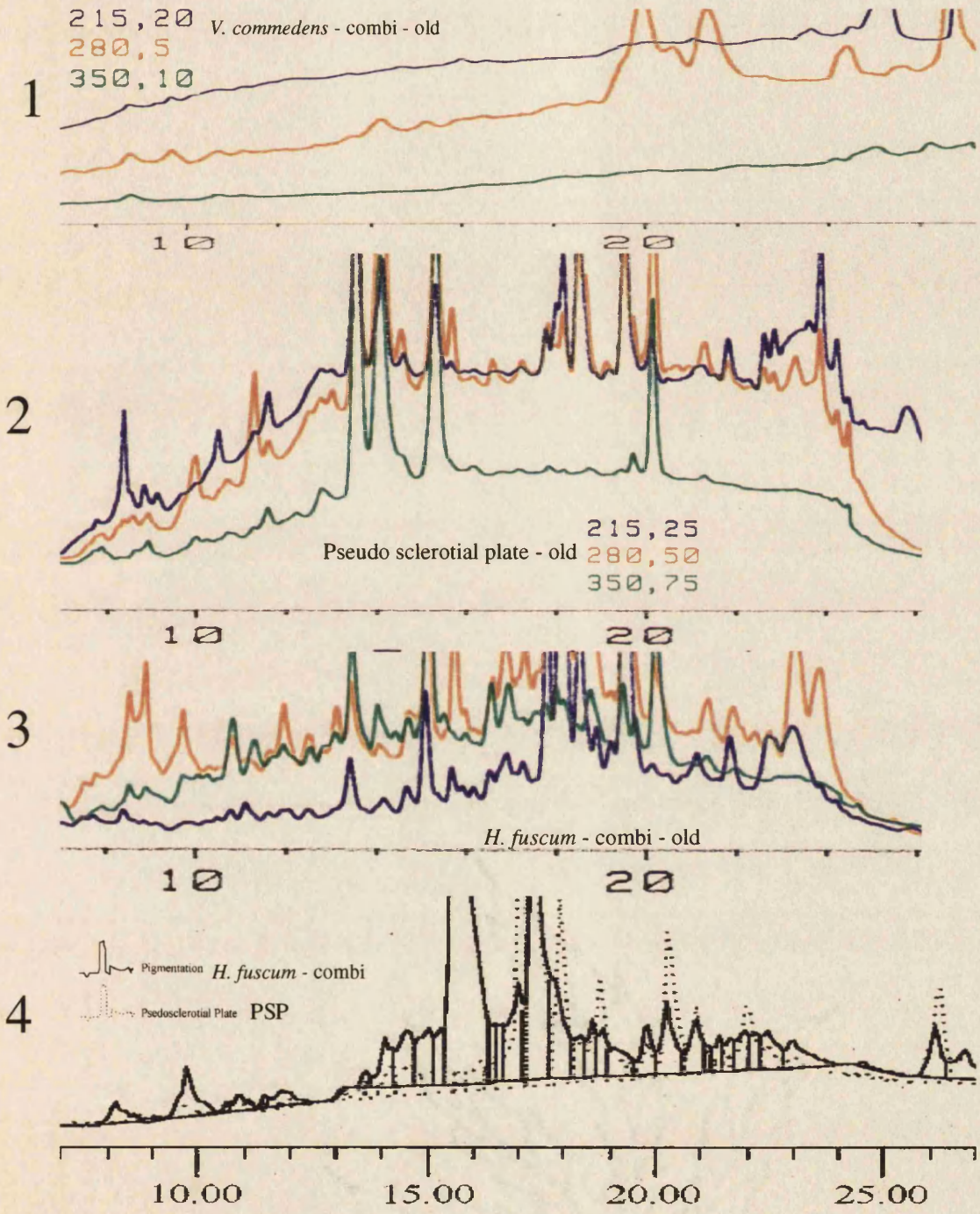


Figure 5. 33 Combination culture H.P.L.C. metabolite profile comparisons (2 runs with 4 samples) between (1) *V. commedens* , (2) PSP zone, (3) *H. fuscum*, and (4) an initial comparison between PSP and *H.fuscum* at 250 nm λ which initiated further work. Retention times are displayed on x-axis.

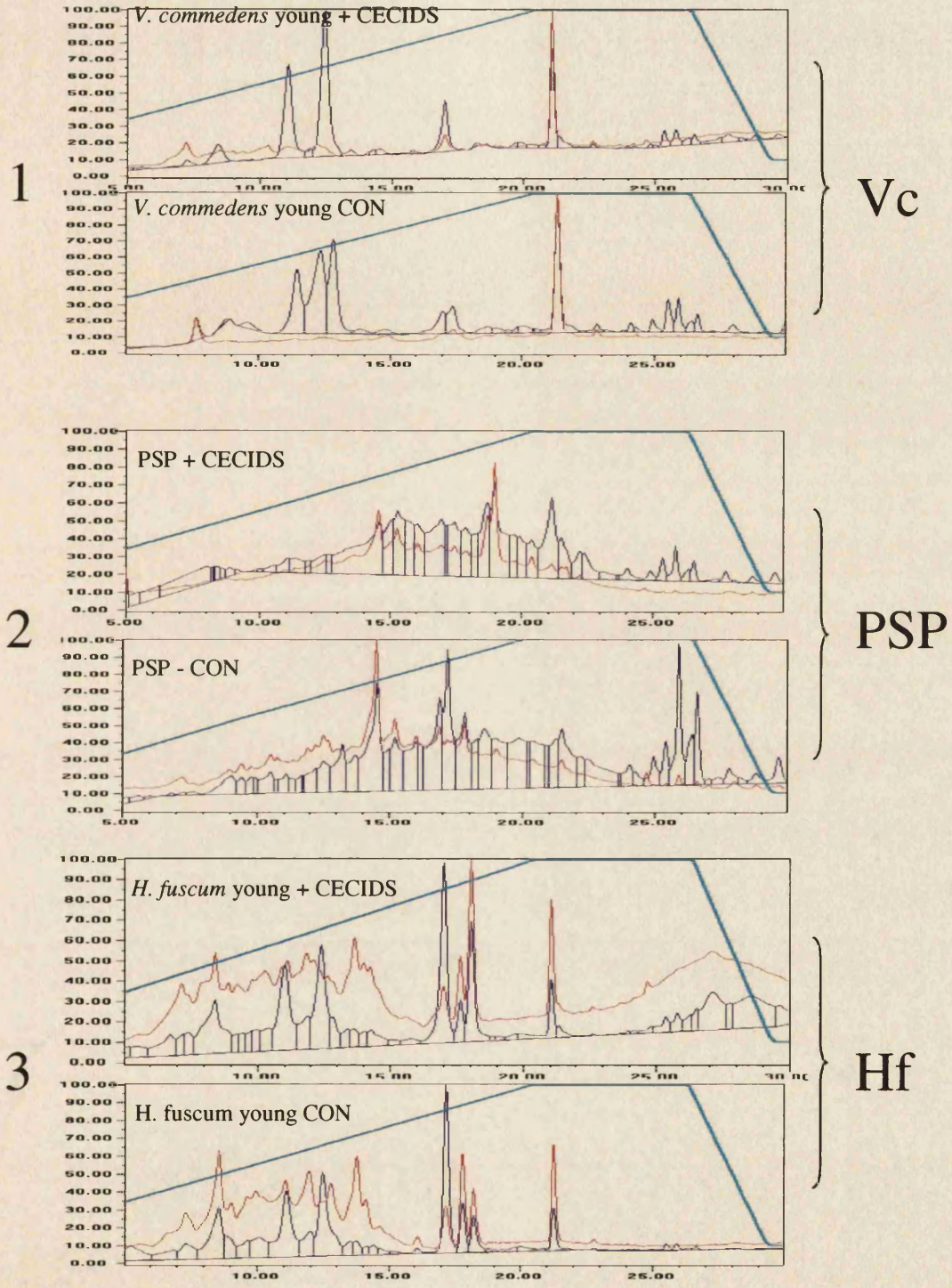


Figure 5.34: Effect of cecids; HPLC metabolite profile comparisons from same run of combined culture extracts (6 interactive fungal samples) with and without cecids; (1) *V. commedens*, (2) P.S.P. and , (3) *H. fuscum*.. Red = 280nm, Blue = 210nm.

5.4 Discussion

The results presented in this chapter regarding radii of foraging loops and positions of larvae in interactive cultures demonstrate that PSP zones strongly influence cecid distribution and foraging. As mentioned in chapter 1, the distribution and foraging trajectories of animals are the scale of development that most strongly parallels the foraging and assimilating structures of plants and fungi. Results regarding pigmentation rates and production of PSP zone shows that cecids provide enhancing influences on the development of the synergistic (non-additive and reactive) aspects of mycelial interactions. There is good evidence provided by PSP data for developmental feedback between cecid larval orientation behaviours, fractal PSP-centred foraging patterns and enlargement of PSP zones between interactive mycelia of basidiomycete and ascomycete fungi. In addition, there is HPLC evidence that suggests that the physico-chemical properties of PSP zones might be synergistically produced (Haken 1980) in a chemically reactive and non-additive sense, from the mycelial ingredients that combine at sites of PSP emergence. There is also HPLC evidence that suggests that PSP chemical properties are seeded by chemical initial conditions specific to particular fungi, and, as such, are specific to their environment. It is also clear from tissue printing that the middle of PSP zones are relatively free from peroxidase enzymes and free radical producing H_20_2 and are flooded with *H. fuscum* derived antioxidants. From tissue printing results it can also be ascertained that **PSP zones contain *H. fuscum* derived catalase activity which releases oxygen bubbles, perhaps responsible for gaseous PSP blisters observed in some cultures and providing some physical potential for structural dynamics, such as the emergence of cracks and ridges within PSP, which could accommodate the observed tight clusters of paedogenic larvae.** It is also clear from HPLC results that the events that occur in freshly forming PSP at the edges of Petri dishes are very different from those in older more stable PSP at more central and older regions of culture plates. Combined HPLC and tissue print evidence suggests that the metabolic events that occur in the midst of freshly forming PSP zones, in comparison with oxidatively stressful areas in fungal mycelia to either side, may provide refuges from oxidative stress. These freshly forming oxidative stress free "skins" between fungi are created by the same chain of events that occur at a different scale as a PSP structure ages and widens and becomes left behind from the metabolic activity which gave rise to it. It seems that the more mature and wider the PSP zone is, the less metabolically hostile its chemical properties and the more stable its physical properties are likely to be. This process of being left behind from wave-like interactions in physico-chemical environments in space and time (4 dimensions) is topologically very similar to the way in which cells develop in animal tissues. In this way, the cecids could be regarded as acting in a similar way within mycelial systems to how free radicals might act in the developmental pathways of other organisms. Not only do the cecids and free radicals disrupt and damage the living boundaries of the systems in which they operate, but if handled correctly and perhaps even accommodated, they may possibly provide a way in which development can be directed towards the most appropriate outcome in any particular chemico-physical environment. There is evidence from HPLC and tissue-lysis printing that cecids provide a transport mechanism by which enzymes and chemicals may be transported across PSP zones from one mycelial system to another. This must alter the development of the chemico-physico properties of the PSP zone, which then affects the way in which a mycelium interacts with surrounding conditions, be they biotic or abiotic. At a finer scale, with image analysis of mycelial zones, the cecids are shown here to be capable of altering the mycelial insulation that occurs around *V. commedens* hyphae. In this respect the cecids seem to affect hyphae in a similar way to free radicals albeit at a vastly

greater scale. It is known that the enzyme system used by cecids to insulate themselves, for example in forming a hemi-pupa, uses peroxidase and H_2O_2 mediated pathways in order to cross-link and thicken the chitin and protein residues of the insect cuticle matrix. So there are two possible explanations for the increase of H_2O_2 and peroxidase left in cecid trajectories. Firstly, these substances could be produced by the cecid larvae and rubbed off the cuticle as they move through mycelium, or secondly, they could be produced by *V. commedens* and imported into *H. fuscum* by cecid foraging. Cecid larvae from the field also carry spores of fungi which germinate and grow in PSP zones and which seem to provide an extra potential for physical ridges and cracks to occur within the PSP structures in agar dishes. The growth of certain fungi in PSP zones has been noticed before (Rayner 1976, Rayner 1992 b). This "poppies in Flanders" effect supports the idea that interactive relations provide surfaces available for other species as niche space (Rayner 2000).

Despite the apparent symmetry in mechanism of cecid-mediated enzyme and chemical transport, there is an asymmetric effect of this mechanism on the biochemistry of recipients of this reciprocal exchange in terms of H_2O_2 and antioxidants. This biochemical asymmetry, induced it seems by cecids, at a greater scale may result in the reduced ability for *V. commedens* to invade *H. fuscum*. Furthermore, the amount of pigmentation of *H. fuscum* is enhanced by cecid activity. Perhaps some of the pigments in *H. fuscum*, PSP, and other Ascomycetes are actually antioxidants, as are chlorophyl and beta-carotine in plants (Bakon 1998, Brown 1981, Cox 1987, Crawford 1992). As suggested in chapter 1, the Ascomycotina are particularly adapted to exhibiting chemical versatility (Crowe 1997). It is thought that Ascomycetes have a particular strategy for dealing with increased oxidative stress, which may explain why catalase activity played a role in the *H. fuscum* but not *V. commedens* mycelia. Because Ascomycetes are less able to seal their boundaries than Basidiomycetes, they have evolved by opting to let free radicals into their system and then swamp them with polar antioxidants and peroxisome-contained catalases (to mop up free radicals and to break down any H_2O_2 into water and oxygen) within the hyphal protoplasm. Ascomycetes thus tend to get metabolically wetter during their interactions with the environment, which perhaps is why we find Ascomycetes adapted to live in, and perhaps even slightly contributing to much wetter forest environments than those of the dry-tolerant Basidiomycotina. Conversely, it is thought that the basidiomycete propensity to excocytose H_2O_2 and peroxidases (Rothchild *et al.*1998 a,b) outside their hyphal walls at any signal of oxidative stress is part of an environment-triggered feedback process which helps to insulate hyphal walls through the peroxidase-mediated release of free radicalised polyphenolics and flavenoid residues which help to build and to cross-link chitin and other fibres into the wall fabric. Any further production of H_2O_2 by such free radical activity may also effectively "dry up" the local hydrative environment by removing free water molecules, which causes further insulation of hyphae in a positive feedback cycle. In this way, Basidiomycetes seem to do the opposite to Ascomycetes when they encounter others in the environment. Paradoxically, it may be this tendency to cause local extra-hyphal chemical "dryness" that enables Basidiomycetes to utilise enhanced insulative physiological development and survive drought much better than Ascomycete mycelial systems. It also enables Basidiomycetes to exhibit their full mycelial dexterity and turn hyphae into collective plumbing networks capable of shunting protoplasm and water over long distances of dryness to sites where growth occurs. However, the strength of the basidiomycete system is also its great weakness because there exists a potential for excess "free" water to reverse this feedback process and reduce hyphal walls, once impermeable to free radical attack, to become thin, flexible and dangerously permeable. All this is advantageous as long as the Basidiomycete does not meet a chemically versatile Ascomycete in this wet environment. If the Ascomycete has enough water supply, it potentially has an advantage over the basidiomycete strategy

because it can utilise its chemical versatility to defend any attack with antioxidants, catalase and production of PSP zones. However this apparent advantage in favour of the Ascomycete is lost once the interaction dries up.

The combination of tissue printing, HPLC and laboratory cultures has enabled at least part of this intriguing dynamic interplay to be seen at several scales simultaneously. Also, the addition of cecids to these interactions has enabled an understanding to be reached as to what happens when an already dynamic system is perturbed. **There are many physical systems where perturbation effects lead indirectly to changed boundary conditions and resultant emergence of changed patterns. I believe that we have here an example of such complexity in a biological system. Having considered the reciprocal effects of cecids, ascomycete and basidiomycete fungi with PSP we may now ascertain the effect of fungal inter-specific interactivity, as opposed to non-interactivity, in influencing the potential for the emergence of particular patterns. This is the topic of chapter six.**

CHAPTER 6: LABORATORY STUDIES OF INTERACTIONS BETWEEN CECIDS AND FUNGI FROM DECOMPOSING HAZEL WOOD, III: COMPARISONS BETWEEN INTERACTIVE AND PURE CULTURES

6.1 Synopsis

Chapter six compares and contrasts interactive and pure cultures where, up to now, they have been regarded separately. There are some new experiments conducted for this purpose. In addition the results from chapters 4 (bipartite interactions) and 5 (tripartite interactions) are compared to draw out the changes, which take place with heightened interactivity of the *Brittenia-Hypoxylon*-PSP-*Vuilleminia* system.

6.2 Introduction

This chapter presents additional experiments designed to help interpret, contrast and compare previous results of pure fungal, bipartite cecid-fungal feedback - as presented in chapter 4, with interactive fungal, tripartite cecid-fungal feedback - as presented in chapter 5. The previous two chapters have separately considered the effects of cecids and fungi on each other in monoculture (chapter 4) or interactive culture (chapter 5). This chapter seeks to build a synthesis from all the laboratory work presented so far. This is thus the third and final of the series of laboratory work chapters 4, 5,and 6. Chapter 4 presented work based on pure single culture systems. Chapter 5 presented work on combined interactive cultures. Differences and similarities between the two culture systems presented here could increase our understanding of the constitutive roles that interactions play in influencing cecid larval development and how feedback affects fungal production of deeply pigmented pseudosclerotial plate (PSP) zones. Such understanding may enable the quality of interactive parameters to be described synergistically, leading to emergent patterns of cecid-fungal-PSP complexes. The term synergistic, in this context, means that there are PSP-specific metabolites arising from tripartite inter-specific interaction, whose combined chemical and physical polymeric properties are greater than the sum of the constituent de-polymerised metabolic precursors found in non-interactive monocultures. As such this metabolic response to being in a state of interactivity describes a non-additive function with novel, unanticipated or enhanced properties, as attributed to classical synergistic states (Haken 1980).

Presentation and interpretation of differences and similarities between the two culture systems occurs in this separate chapter for two reasons. Firstly, so that previous chapters could focus on interesting events with respect to bipartite and tripartite interactions, to which they were particular. Secondly, so that special attention could now be given to reciprocal effects of cecid perturbation triggered feedback in the mycelia of fungi in complex dynamic (tripartite) as opposed to simple dynamic (bipartite) systems. The results from previous chapters 4 and 5 are summarised for the purpose of this comparison in table 6.1 overleaf.

TABLE 6.1 Differences between pure (bipartite) and interactive (tripartite) cultures: Key: ↑ = enhanced phenomenon, ↓ = reduced phenomenon, **myc** = mycelium, **speed** = rate of development, **diffuse** = diffusivity of zone (i.e. lack of sharpness), **invas** = invasions by other fungi, **Anti-ox** = antioxidant, **Peroxid** = peroxidase activity, **Catalase** = catalase activity, **H_2O_2** = H_2O_2 production, **Protein** = Total protein production, **Non-polar** = peaks produced at right hand end of HPLC elution specra, **Polar** = peaks produced at left hand end of HPLC elution spectra, **Unique** = peaks which are only found in this zone, **Gt** = generation time, **Gno** = offspring per generation, **Aggr** = aggregation of larvae in local spots, **topigment** = attraction of cecids to pigmented areas, **under hyphae** = attraction of cecids to spots underneath insulated mycelium, **Asymm** = Asymmetry of colony shape.

Status	bipartite	bipartite		tripartite	tripartite	tripartite
Species	*H. fuscum*	*V. commedens*		*H. fuscum*	PSP	*V. commedens*
Effects of cecids on fungal culture	Pigment ↑ Integration ↑ Unpigment ↓ Asymm ↑ Area ↓	Aerial myc ↑ Integration ↑ Asymm ↑ Area ↓		Pigment ↑ Flatening ↑ Aerial myc ↓ Asymm ↑ Area ↑ H_2O_2trails ↑	Speed ↑ diffuse ↑ Trails cut ↑ Asymm ↑ Area ↑ 2° invas ↑ Cracks ↑ Ridges ↑ Bubbles ↑	Aerial myc ↑ Invasive chords ↓ Asymm ↑ Area ↓
Effects of cecids on fungi –t.prints	Anti-ox ↑ Peroxid ↓ Catalase ↑ H_2O_2 ↓	Anti-ox ↑ Peroxid ↓ Catalase ↑ H_2O_2 ↓		Anti-ox – Peroxid ↑ Catalase ↓ H_2O_2 ↑ Protein –	Anti-ox ↑ Peroxid ↑ Catalase ↓ H_2O_2 ↓ Protein ↑	Anti-ox ↑ Peroxid ↓ Catalase – H_2O_2 – Protein –
Effect of cecids on HPLC	Non polar ↑ Polar ↓	Non polar ↑ Polar ↓		Non polar ↑ Polar ↑	Non polar ↑ Polar ↓ Unique ↑	Non polar ↑ Polar ↑
Effects of fungi on cecids	Gt ↑ Gno ↓ Loop radii ↓ Aggr ↑ to pigment ↑	Gt ↑ Gno ↓ Loop radii ↑ Aggr ↓ Under hyphae ↑		Density ↑ Loop radii ↓ Aggr ↑ Topigment ↑	Birth ↑ Density ↑ Loop radii ↓ Aggr ↑ Topigment ↑	Density ↓ Loop radii ↑ Aggr ↓ Under hyphae ↑

In light of table 6.1, further work is presented in this chapter to clarify any differences and similarities attributed to states of simple (bipartite) and heightened interactivity (tripartite) cecid-fungal systems. Moreover, work here aims to differentiate between constitutive and synergistic effects by utilising an integrated tissue-lysis-printing HPLC thin-layer chromatography (TLC) protocol in concert with further interactive and pure culture experiments.

6.3 Materials and methods

6.3.1 Split plate culturing to determine the influence of PSP zone

To answer the question: "**How important are pseudosclerotial plate (PSP) zones, as distinguished from the presence of 2 mycelia of different species, in explaining the differences observed between pure and interactive cultures**?" an experiment was carried out with combined culture but no interaction. One 5 mm diameter mycelial plug was aseptically inoculated into each side of split culture plates in which the Petri dish was divided in two by a plastic wall which cecids could negotiate whilst *H. fuscum* and *V. commedens* could not (Spencer 1998). This meant it was possible to grow both fungal species in the same Petri dish with effectively no interaction or PSP zone - other than that being

generated over time by cecid vectoring of fungal protoplasm and metabolites. The fact that cecid larvae were free to forage over both mycelial domains of split culture plates meant that they might still gain dietary and other influences from combined culture, without the influence of a PSP zone. The combined *H. fuscum* and *V. commedens* without PSP split plates were tested against split plates with pure *H. fuscum*, *V. commedens*, *Micoacea uda* and *Phanerochaete velutina* - the latter two fungi also being isolated (chapter 4) from the field site (chapter 3).

6.3.2 Repli-plate culturing experiment

A 25-well interconnected repli-plate (with small cuts melted into the sides to allow mycelia and cecid larvae to migrate from well to well) was inoculated with high (2 % malt) and low (no malt) agar nutrients in a checkerboard array. *H. fuscum* was inoculated into the centre of each well. With the aid of a heat-free light source, cecids were counted in each partition on days 50 and 80 over a 90-day time course.

6.2.3 Time-lapse filming

To test ideas from trajectory measurements in chapter 5, trajectories in pure and interactive fungal cultures were measured from a time-lapse film. This film was made using a 16 mm Bolex film camera linked to a Bolex time delay switch with two outputs set to operate every 10 minutes. The first output operated a lamp, which came on a split second before the second output opened the shutter of the camera. The camera was mounted on a Bolex camera stand with a focussed macro-lens, and positioned vertically above a perspex cylindrical filming chamber with a cellophane window. The time-lapse film method was adapted from the method for fungal Petri dish and repli-plate filming developed by Sharry (Watkins and Sharry 1996). The hypothesis being tested here was that the mother and first-instar larvae moved at considerably different speeds, and also distributed themselves differently in interactive and pure cultures. Two one month films were made, each consisting of 12 minutes of 16 mm film footage, one for pure *H. fuscum* and cecids and the other for interactive cultures of *H. fuscum* and *V. commedens* treated with cecids. Both films were converted to VHS videotape and then digitally converted and compressed into two minutes of CD-ROM moving image from which data analysis could proceed from selected frames (see Appendix 3).

6.3.4 Cecid larval velocity experiment

The velocity of larval foraging was measured from time-lapse films by converting 16 mm film to VHS videotape by filming movie projections with a high quality video camera in a dust-free room. The VHS videos were then converted into digital AVI and MOV files for Windows formats using a video - digital decoder codec for Windows. Separate frames of the films were saved as JEPG images. By measuring the changes in pixel co-ordinates of individual larval trajectories between successive film images on the screen, the velocities of selected *B. fraxinicola* cecid larva could be calculated (Donnelly, Boddy and Wilkins 1999).

6.3.5 Larval length measurements

Lengths of *B. fraxinicola* larvae were measured in both single pure culture and combination interactive culture using a binocular microscope with an eyepiece graticule calibrated at x 40 magnification to measure to the nearest 0.1 of a mm.

6.3.6 Thin Layer Plate Chromatography

6.3.6.1 Preparation of samples on TLC plates

To compare the different oxidatively reactive components of fungal protoplasm between single and combined cultures, thin layer plate chromatography (TLC) was used with an adapted method from Paterson and Bridge (1994). Two sizes of TLC plates were used. Initial runs used small sized 10 by 10 cm glass supported plates with a silica gel matrix to depth of 0.6 mm. Large sized plates were 20 x 20 cm Merk thin layer plates composed of a fixed silicon matrix (silica gel 60) to a thickness of 0.2 mm on aluminium support. These two types of TLC plate were spotted with ethyl-acetate samples freshly prepared for HPLC analysis (see chapter 4). These samples were composed of mycelial plugs shaken overnight in light free glass cuvettes with HPLC grade ethyl acetate. The plate "spotting" protocol involved placing an HB pencil line 1.5 cm above the bottom of the plate and placing a cross for the point at which each sample was to be loaded into the matrix. The method to "spot" samples was to take one fresh capillary tube for each sample to be loaded and dip it into the cuvette until it filled with solution, before transferring the solution to the correct loading point on the TLC plate. To keep the loading as tight and focussed as possible, the capillary tube was removed as soon as the slightest amount of contact had been made, to prevent excessive spreading of sample radially from the loading point. Once the ethyl acetate had evaporated from the sample's spot, thus fixing the sample of protoplasmic metabolites into the matrix, the capillary tube could be re-lowered to make contact with the matrix again, and so on until the capillary tube needed re-loading with sample. Twenty capillary tube re-fills were loaded into the sample loading points using this method, thus loading a total of 150µl of each sample. To speed up the whole process, a hair drier was used to increase the rate of solvent evaporation so that the rate of applying spots could be increased. The final result was a series of equally spaced, highly concentrated and focussed spots of labelled samples across the bottom of each TLC plate.

6.3.6.2 Running and developing TLC plate chromatographs

Once fully loaded, the plate was run by standing the plate vertically in a covered glass tank filled to a depth of 1 cm with chloroform-acetone-propan-2-ol in the Ratio 85:15:20. The solvent front (mobile phase) was allowed to migrate by capillary attraction up the silica matrix (stationary phase) until it had climbed to a height 3 cm from the top of the plate. The plate was then removed and viewed under wavelengths of normal white-light and then of UV-light with protective eyewear. Pencil markings were then drawn around each band under UV. Plates were then sprayed with either a free radical solution of 1-Diphenyl-2-picryl-hydrazol (DPPH) made with 80 µg of DPPH in 25 cm^2 absolute ethanol (to detect bands of antioxidants), or anisaldehyde reagent made with 0.5 ml anisaldehyde in 9 ml ethanol containing 0.5 ml conc. H_2SO_4 and 0.1 ml glacial acetic acid (to detect sterols, phenols, terpenes, and myco-toxins). The plates sprayed with anisaldehyde reagent were then placed in a pre-heated oven to develop for 10 minutes before viewing again under normal and UV-light. A third spray was ferrichloride (Sigma) TLC developing reagent to detect complex aromatic metabolites with double bonds (Jark, Funk and Fischer 1990). Spraying was conducted in a cardboard box within a fume cupboard. Small-sized TLC plates were first viewed under white and UV light, then sprayed with ferrichloride and then viewed again under white and UV light. Large plates were first viewed and then sprayed with anisaldehyde or DPPH sprays. Pencil markings were drawn with a soft but sharp HB artists pencil around all bands and labelled with the spray used and the wavelengths of light

(normal-white, UV 254 nm or UV 366 nm). Banding patterns were finally investigated for similarities and differences between samples. As retention fractions (Rf) approach a value of 1, proportionately less of the fraction is retained by the stationary phase matrix, and more of the fraction is destined to form a band higher (non-polar end) in the banding pattern towards the position of the solvent front.

6.3.7 HPLC analysis

6.3.7.1 *Brittenia fraxinicola* and *Corylus avellana* metabolites

Ethyl acetate samples were prepared of *B. fraxinicola* internal and external metabolites. External cuticular-linked metabolites were prepared by removing 20 cecid larvae from interactive experimental cultures and washing them in 75 % ethanol for 20 seconds, then 1 minute in 2 % formaldehyde and 20 seconds in MilliQ water, before placing in a glass cuvette. 9 cm^3 ethyl acetate was added and the extract made by shaking overnight in a light-free box (see chapter 4). Internal metabolite samples of *B. fraxinicola* larvae were prepared by following the same procedure before lysing larvae with a clean glass rod in the ethyl acetate solvent. An additional extract was made in the same way with 5 g fresh triple layered hazel bark taken from a cecid colony in the field site. Using Gilson software, previous runs of fungal metabolites were compared on the same axes to be able to compare peak retention times between pure and interactive culture samples.

6.3.7.2 HPLC comparisons of pure and interactive cultures

Following the HPLC protocols detailed in chapter 4, two new runs were made specifically to compare pure fungal and interactive culture metabolites at the same time. 20 samples were loaded into the column as set up in the methods of chapter 4. These comprised 2 repeats from each of the 10 following zones: 3 interactive zones without cecids, 3 interactive culture zones with cecids, 2 pure culture zones with cecids, and 2 pure culture zones without cecids.

6.3.8 Cecid foraging trajectories and body lengths

The same methods were employed to compare cecid foraging loop radii across zones of pure and interactive fungal cultures as were detailed in chapter 5. Body lengths (mm) were also compared between parallel interactive and pure culture systems by measuring cecids with a binocular microscope fitted with calibrated eyepiece graticule.

6.3.9 Statistical analysis

Paired t-tests were used on parametric normally distributed data with samples of $n>9$, whereas Mann-Whitney tests were used where sample size was lower than 9 (Wheater & Cook 2000). Correlation analysis was carried out between variables where appropriate. Statistical analyses were carried out using Minitab for Windows software.

6.4 Results

6.4.1 Split culture and repli-plate results

Figure 6.1 shows results of matrix plate cecid population dynamics according to changes in intra-specific mycelial development, and below that, results of experiments to compare split culture plates compared to pure cultures (these pure cultures were also grown in split plates as a fair comparison). The variation of *B. fraxinicola* body length in split plate experiments was not significantly greater than pure culture body length. However, despite the lack of pseudosclerotial plate (PSP), the overall population size reached by *B. fraxinicola* in split culture plates was significantly higher than in pure cultures.

6.4.2 Cecid body length measurement results

Results for interactive cultures and pure cultures are shown in figure 6.2. In contrast to the situation with split plate cultures with no PSP, a significant increase ($P<0.001$) in body length can be seen with interactive PSP present. The frequency distribution for interactive culture larval lengths (3) shows a bimodal distribution (blue). The smaller sized peak is not significantly different to the single peak found in mono (pure) cultures of *H. fuscum*, and *V. commedens* (grouped in red) but the second interactive culture peak lies significantly to the right of the pure culture data set. This latter data is significantly longer ($P< 0.0001$) causing the whole data set for interactive cultures to be of longer mean length (2). In a repeat experiment shown in graph (1), a detailed distinction of where measurements were recorded is shown. Graph 1 shows that larval lengths were significantly smaller in pure culture plates than in combined interactive plates. Also graph 1 shows larval lengths in interactive PSP zones were significantly smaller than in other two interactive culture mycelial domains. Also graph 1 shows that lengths at the surface ("combi surf") of interactive cultures were not significantly different to those at the base ("combi sub") of the Petri dishes - although there is an interestingly greater amount of variability in cecid lengths at the surface as shown by standard error bars.

6.4.3 Cecid foraging trajectory results

Figure 6.3 (5) & (6) show difference between pure and interactive cultures in terms of toroidal foraging loops measured in plates incubated in parallel under standard laboratory conditions. Paired t-tests of data (7) & (8) are displayed showing probability of the variance being due to chance. High significance ($P<0.0001$) is shown between loop radii in different fungal zones, with the smallest radii in the PSP zone. However no significant difference in foraging loop toroidal radius was found between pure and interactive treatments of the same fungi. A repeat experiment measuring loop radii only in interactive cultures (graphs 1 & 2) confirmed that significant differences ($P<0.0001$) in toroidality of foraging trajectories occur between different fungal species, as well as between fungal and hydrophobic domains such as the lid of the Petri dish.

6.4.4 Cecid larval velocity and time-lapse film results

Cecid velocity data is shown in figure 6.4. Appendix 3 is a CD-ROM with the time-lapse films from which data were derived. Appendix 3 can be viewed on any Windows compatible media player software. The first film shows pure *Hypoxylon fuscum* with cecids. Here the smallest first-instar *B. fraxinicola* larvae can be seen rapidly following the edge of the Petri dish rim whilst, half way through the film, a large mother undulates her way across a small bare region of the culture plate at the bottom of the screen. The second film

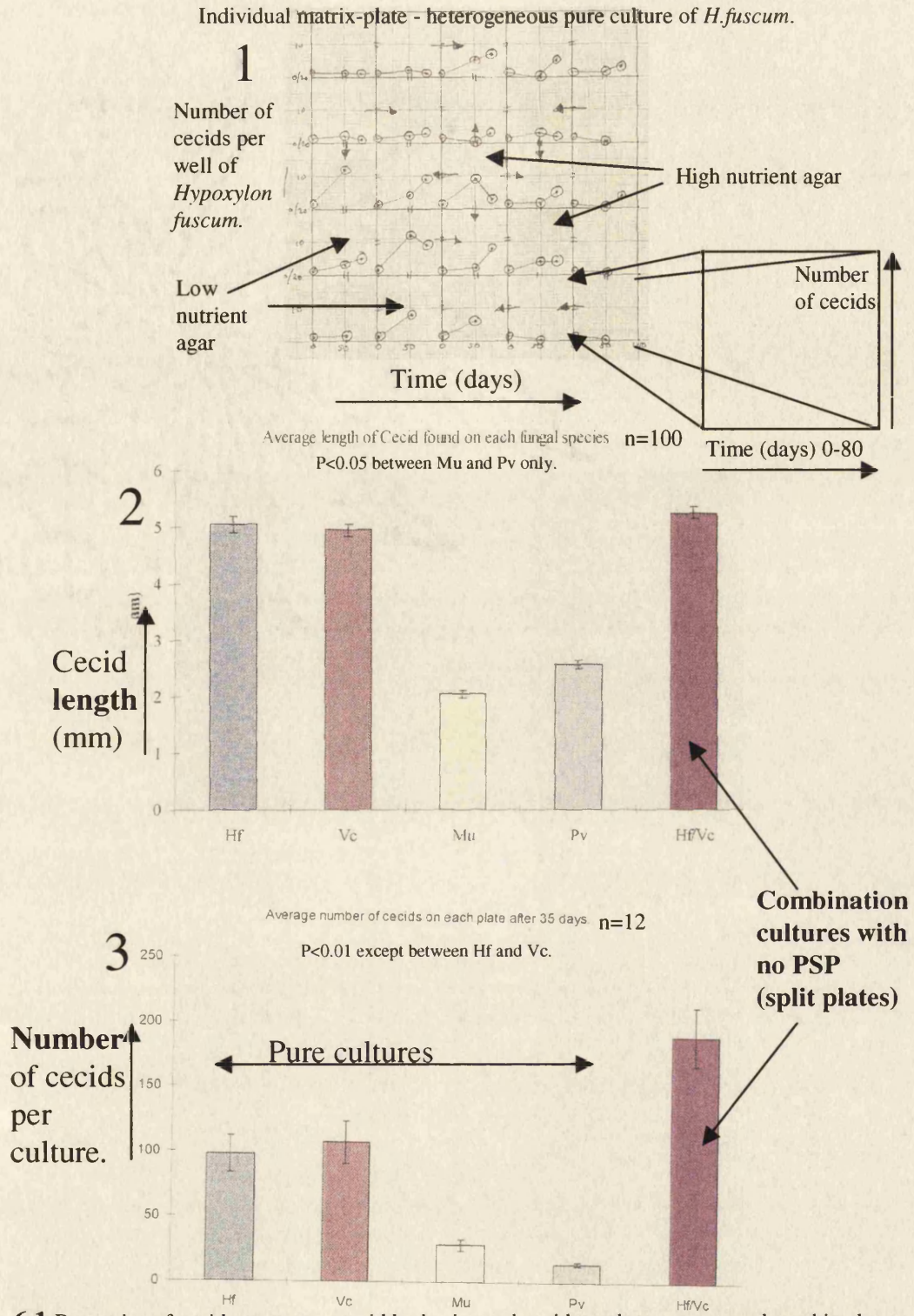
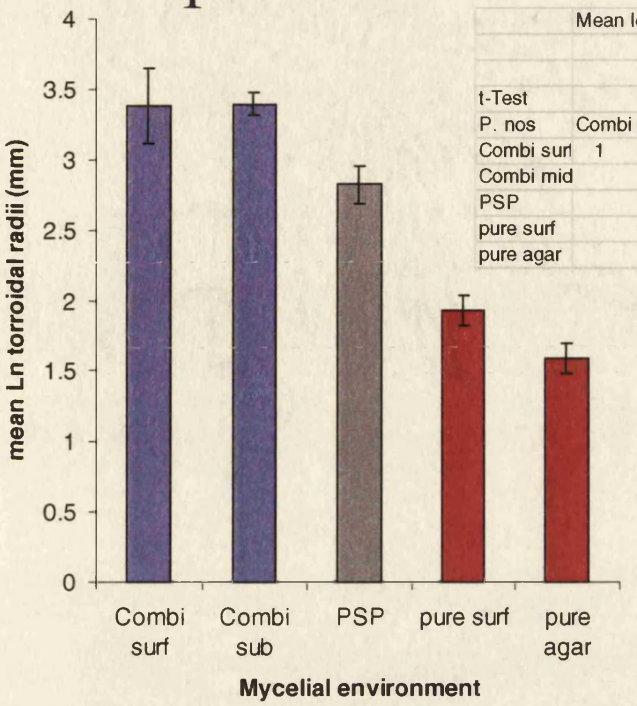


Figure 6.1 Dynamics of cecid movement, cecid body size and cecid numbers on pure and combined cultures. 1 shows cecid movement and population growth in matrix plates of *Hypoxylon fuscum* with high and low nutrient agar in chequer board array. Each of the 25 wells of the matrix plate has recorded in it three data points for number of cecids / well at 0, 50 and 80 days after placing one newly born cecid larva in the centre of each well. Arrows show probable direction of movement from well to well. 2 shows the length of cecids in combination cultures without PSP zone (grown in split petri-plates with plastic divisions) does not alter significantly from mean lengths in *H. fuscum* and *V. commedens* pure cultures. 3 shows emergent cecid populations in split plate combination cultures, which lacked PSP, to be significantly greater ($P<0.01$) than that in the pure cultures. Hf = *H. fuscum*, Vc= *V. commedens*, Mu = *Micoacea uda* and Pv = *Phanerochaete velutina*.

1



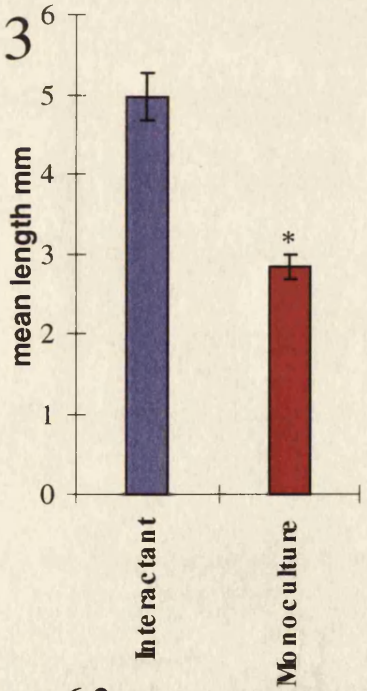
2

Mean length of ceicds in different culture environments.

t-Test

P. nos	Combi surf	Combi mid	PSP	pure surf	pure agar
Combi surf	1	**0.02**	**0.003**	**0.0001**	**0.001**
Combi mid		1	0.661	**0.003**	**0.001**
PSP			1	**0.0001**	**0.0001**
pure surf				1	0.89
pure agar					1

3



4

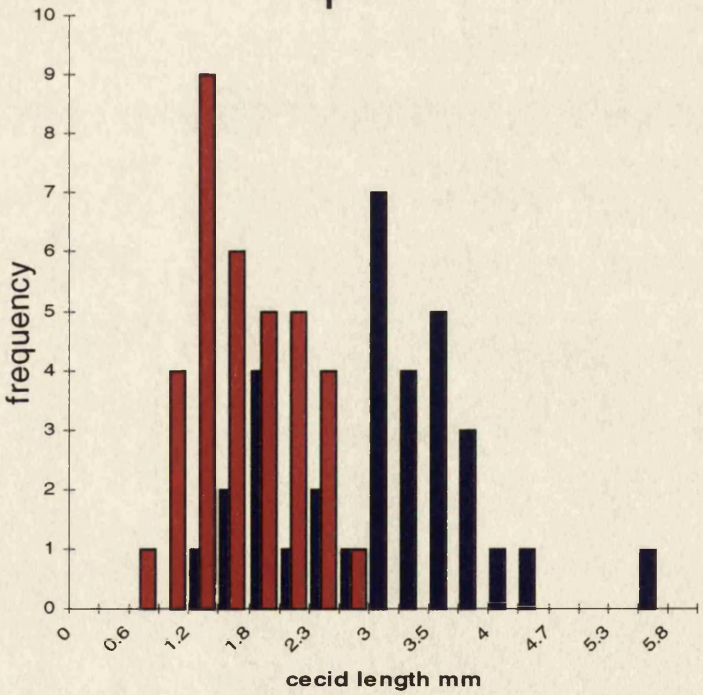
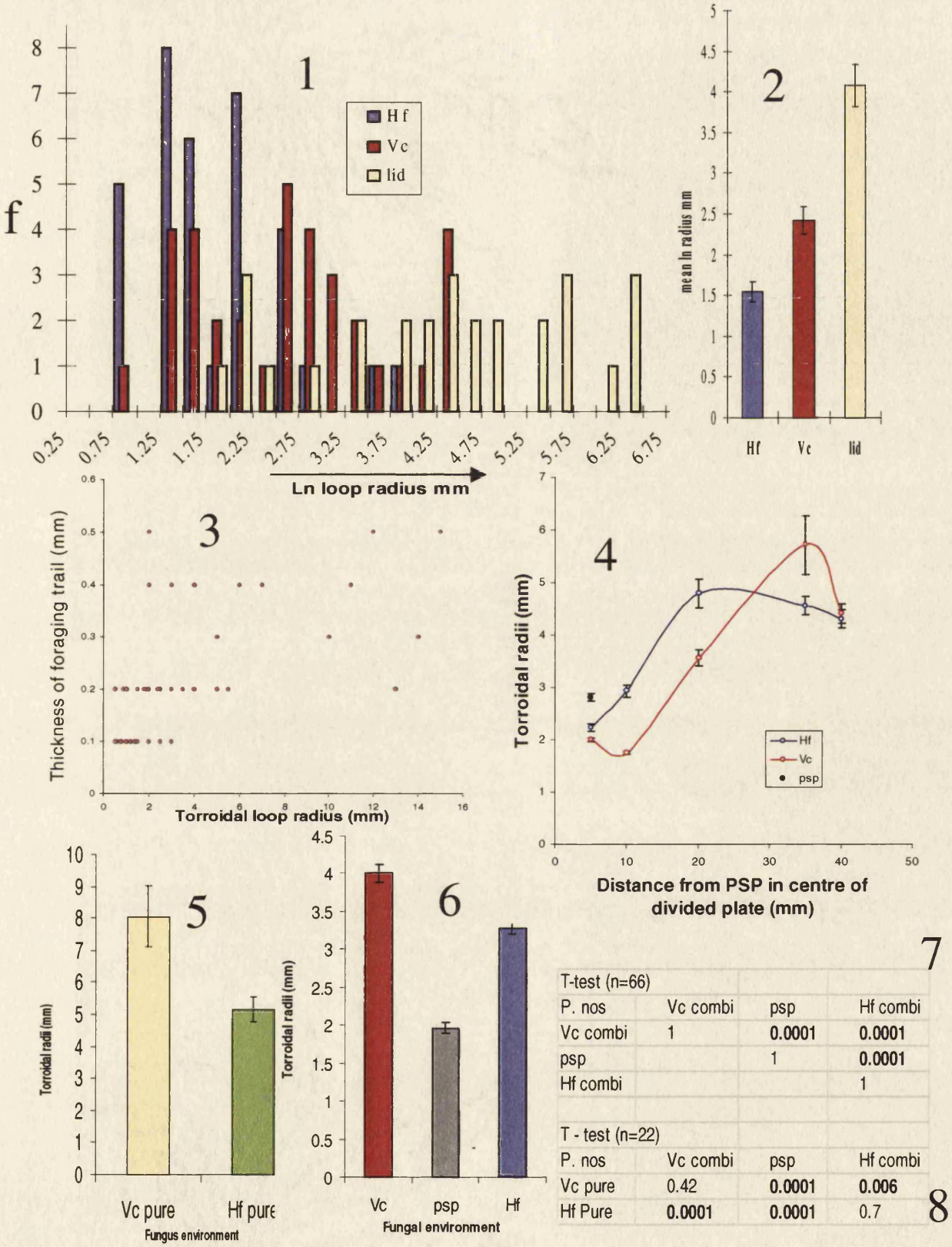


Figure 6.2 Variations in cecid size according to fungal environment; (1) shows the distribution of cecid lengths in each zone of interactive culture. **Key: combi surf** = surface of interactive culture, **combi sub** = underside of interactive culture, **PSP** = pseudosclerotial plate zone, **Pure surface** = surface of monoculure mycelia, **pure agar** = agar with no hyphae. For (1): (n=100), P<0.001 between pure and the rest and P<0.05 for PSP being different from the rest. Standard error bars are shown. (2) shows t-test probabilities for significant difference in plot (1). (3) shows mean length variations overall between interactive and mono-culture mycelia (n=35). Student t-test confirms (3)'s significant difference of means (P<0.0001 asterisk). The raw data for (3) is plotted in (4). Here a bi-modal distribution of larval size on interactive cultures is apparent. The mono-culture measurements were collated for both *H. fuscum* and *V. commedens*. In all the plots of this figure, **blue** = interactive culture and **red** indicates pure culture.



7

T-test (n=66)			
P. nos	Vc combi	psp	Hf combi
Vc combi	1	**0.0001**	**0.0001**
psp		1	**0.0001**
Hf combi			1

T - test (n=22)			
P. nos	Vc combi	psp	Hf combi
Vc pure	0.42	**0.0001**	**0.006**
Hf Pure	**0.0001**	**0.0001**	0.7

8

Figure 6. 3 (1) shows measurements of radii of cecid foraging loops in interactive fungal cultures, (2) as compared in t-test with plot of mean distributions (n=30, P<0.0001) where standard errors of mean are shown. (3) shows relationship between thickness of foraging trail and loop radius (significant correlation P<0.0001). (4) shows variation of foraging loop radius with distance from PSP in centre of plates for Hf (*H. fuscum*) in blue, Vc (*V. commedens*) in red and PSP (in black) where standard errors of means shown. (5) shows loop radii in pure cultures of Hf. and Vc. (n=22), where t-tests show significant difference (P<0.0001). (6) shows loop radii of cecids in interactive cultures of Vc., Hf., and PSP (n=66) where all exhibited significantly different responses (t-test P<0.0001). (7) shows t-test P values of interactive culture distributions whereas (8) shows t-test P. values for pure cultures.

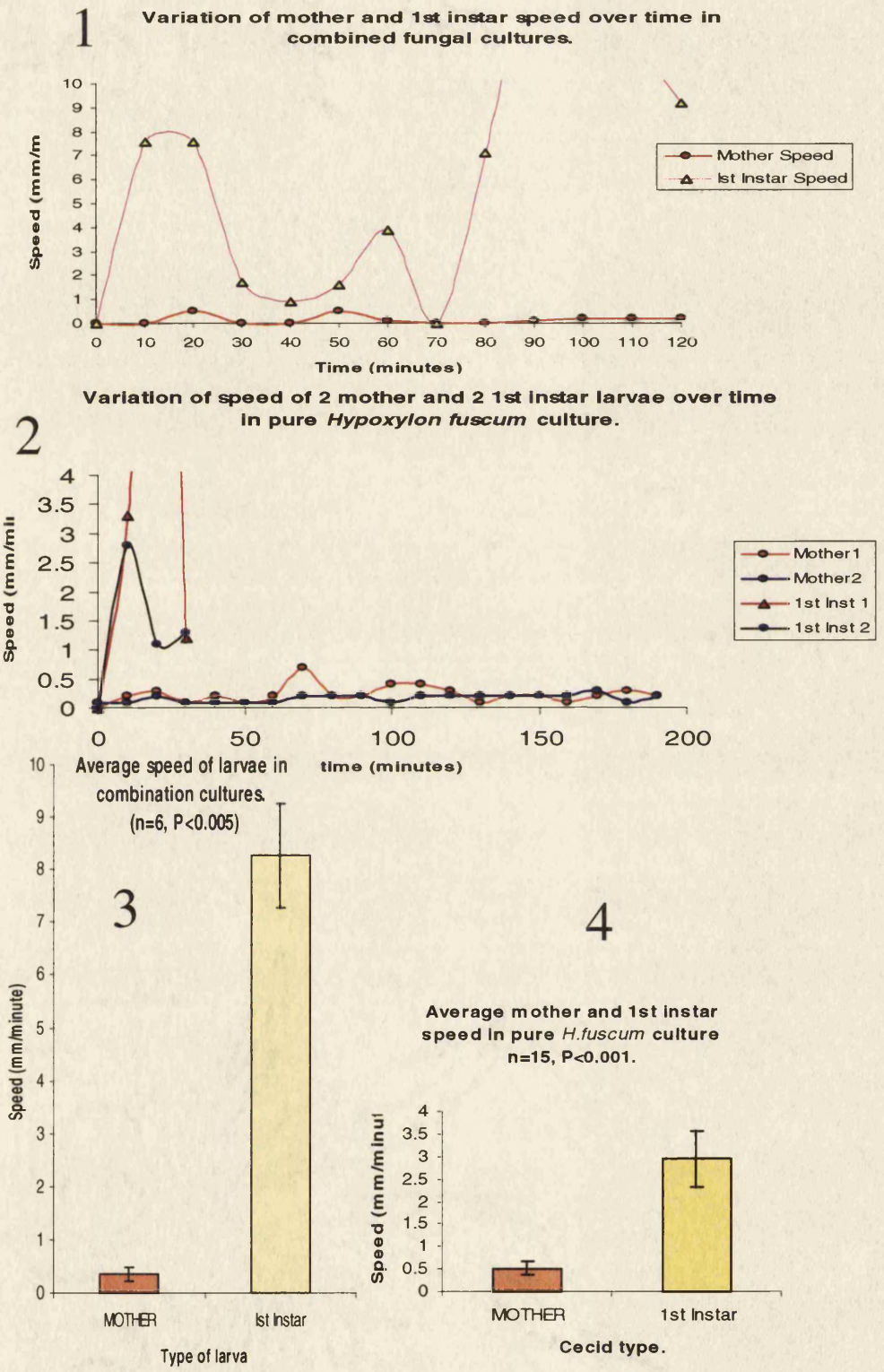


Figure 6.4 (1) Interactive fungal culture larval foraging speeds of mother and new-born over time. (2) Pure culture larval foraging speeds compared to mothers larvae. (3) shows comparisons of mean distributions for interactive culture.(n=6, Mann-Whitney test $P<0.005$). (4) shows comparisons of speeds between mothers and 1st instar daughter larvae in pure culture (n=15, Mann-Whitney test $P<0.001$).

shows a combination culture treated with cecids, which has developed a large PSP zone. Towards the end of this film the Petri dish rim is teeming with rapidly moving first-instar larvae whilst mother larvae move more slowly and sporadically on the PSP zone until paedogenesis occurs. Changes in larval velocity, for combined culture and pure *H. fuscum* are shown in (1) and (2). Periodicity in movement of both mothers and first-instar larvae are similar - moving for 20 minutes, then slowing down or stopping for 20 minutes. The difference in velocity measurements between mothers and first-instars were very large. **Mothers generally moved at less than 1 mm per minute whereas first-instar larvae moved at speeds between 1 and 9.5 mm per minute**. The measurement variances are shown in graphs (3) and (4) to be significantly different ($P<0.005$).

6.4.5 Results at metabolic scale

6.4.5.1 Tissue-lysis printing results

Figure 6.5 shows main trends in comparing interactive and pure culture tissue print assays. Panel 1 shows the effect of interaction to increase the development of *H. fuscum* pigmented mycelium. Panel 2 shows the effect of interaction to reduce free radical presence near the PSP zone. Activity of free radicals is confined to the margins of mycelium. Panel 3 shows the effect of interaction to enhance peroxidase activity *in V. commedens* but not to change peroxidase activity in *H. fuscum*. The peroxidase activity in *V. commedens* stops short at the PSP zone. Panel 4 shows H_2O_2 production. The PSP zone and deeply pigmented associated regions of interactive *H. fuscum* exhibit reduced H_2O_2 production.

6.4.5.2 Thin Layer Plate Chromatography (TLC) results

6.4.5.2.1 Small TLC plate results

Figures 6.6 to 6.9 show TLC plate metabolite banding patterns. The smaller sized TLC plates (figure 6.6), sprayed with ferrichloride, show a banding pattern as follows: TLC plate 1 shows that interaction between *H. fuscum* and *V. commedens* causes *V. commedens* to lose 2 bands visible at UV 366 nm wavelength and *H. fuscum* to gain and also lose a band at UV 366 nm. The upper TLC plate's PSP lane shows a banding pattern at UV 366 nm with no unique bands. PSP bands have more in common, in terms of distance travelled up the solvent front (Rf value), with the banding pattern of *V. commedens* than *H. fuscum*. However, the bottom TLC plate shows a different PSP result where the sample does possess one unique band. This TLC plate also repeats the previous observation that *H. fuscum* both loses and gains a band in it's interactive state.

6.4.5.2.2 Large TLC plate results

Large TLC plate results were different and are shown in figures 6.7 to 6.9. The plates in figure 6.7 were sprayed as follows: the top (1) with anisaldehyde reagent (to test for steroids, phenols, terpenes and myco-toxins) and the bottom (2) with DPPH (to test for antioxidants). Clearly the effect of the sprays has changed the banding patterns in these same samples. The lower TLC plate (2) runs the same samples minus lanes 10-12. The other interesting "global effect" of spraying was that the reactions caused changes to chemicals, which were not themselves the intended targets of the spray. For example, DPPH only clears from its pink colour in areas of antioxidant activity, but spraying the separated metabolites with this free radical caused many more bands to become UV-visible than were previously visible under UV-light. The majority of these compounds possessed no antioxidant activity. This means that the comparisons of banding patterns can only

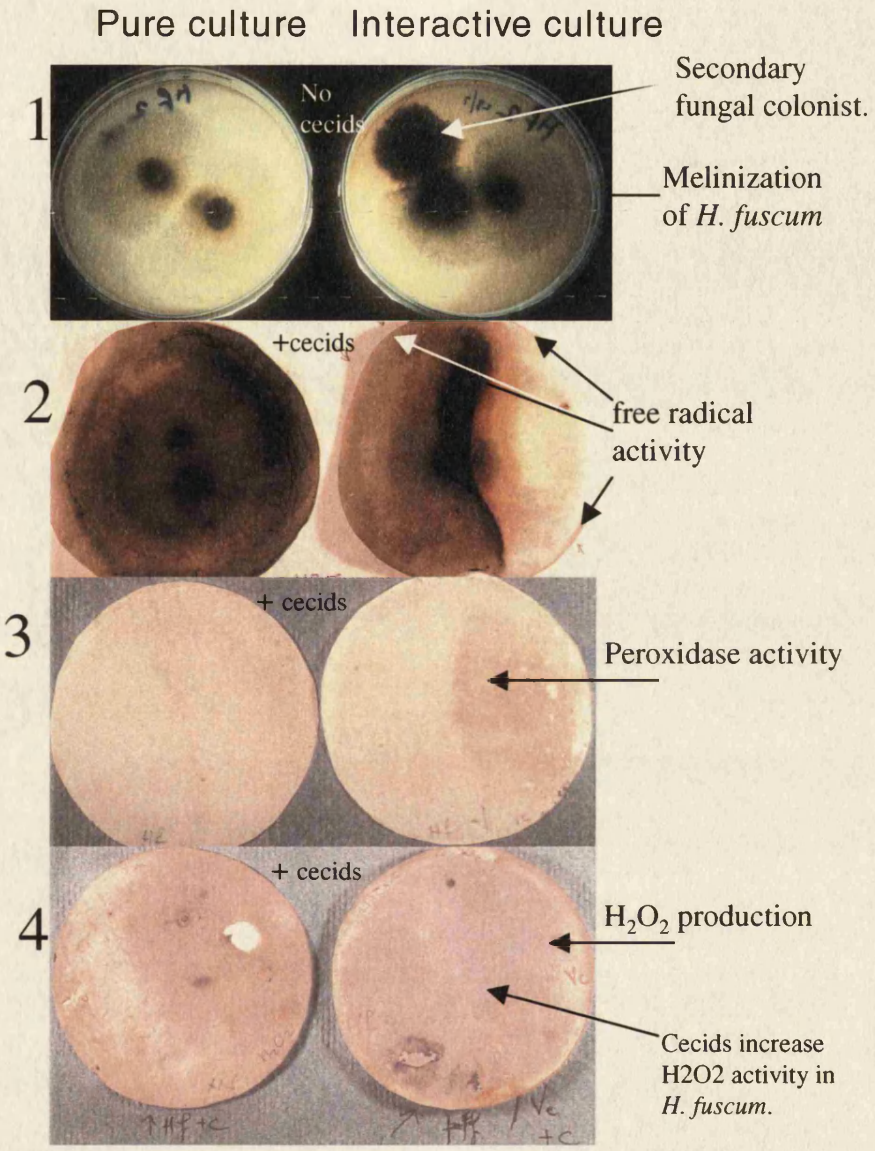


Figure 6.5 Comparisons between pure *H. fuscum* cultures and combination cultures; 1 shows how *H. fuscum* responds, in general, to interaction by enhancing the process of melanisation which is already performed during ageing. 2 shows how in both pure and combination cultures, free radical activity is confined to active margins but is absent from PSP zones. 3 shows *V. commedens* with it's high peroxidase activity in comparison to *H. fuscum*. 4 shows that H_20_2 production is reduced at PSP zones and that cecids distribute H_2O_2 into *H. fuscum* and increase it's action there.

be made between TLC plates with the same spray. The higher up the TLC plate a chemical band is found, the higher the Rf value of that compound, and the less polar (more non-polar) the metabolite is.

Regarding the top TLC in figure 6.7 (1), the anisaldehyde treated plate showed the PSP banding pattern to be most numerous (4 bands in total) with two PSP bands which had not been previously visible under UV light. One of these bands was shared only with interactive state *H. fuscum* and not with the young or single culture *H. fuscum*. The effect of interaction was for *H. fuscum* to gain two bands: one at UV 350nm and one anisaldehyde reactive band. The effect of interaction on *V. commedens* was to lose an anisaldehyde reactive component and gain a component at UV 350nm. The effect of age on *V. commedens* and *H. fuscum* were almost diametrically opposed. As age increased *V. commedens* lost two of its three bands to be left with one, but *H. fuscum* gained three to the initial one; revealing four in total.

Regarding the lower TLC plate in figure 6.7 (2), which was sprayed with DPPH to test for antioxidant production, the largest trend was the PSP zone having the most numerous banding (a total of 13 bands), 5 of which had Rf values unique to the PSP zone and the lower 3 of which were antioxidants. *V. commedens* cultures have the least banding (ranging from 3 to 5 bands). The antioxidants produced by *H. fuscum* and the PSP zone were of low Rf value (polar) whereas those produced by *V. commedens* tended to be of high Rf value (unpolar) - except for the old *V. commedens* and *V. commedens* sample 3 (Vc 3) in lane 9, which was taken from the second isolate of the fungus (see chapter 4) and produced a low Rf value antioxidant in pure culture. (It should be pointed out here that all the results presented so far regarding *V. commedens* and *H. fuscum* have not used second isolates unless specified).

6.4.5.2.3 TLC Interactive fungal trends were as follows

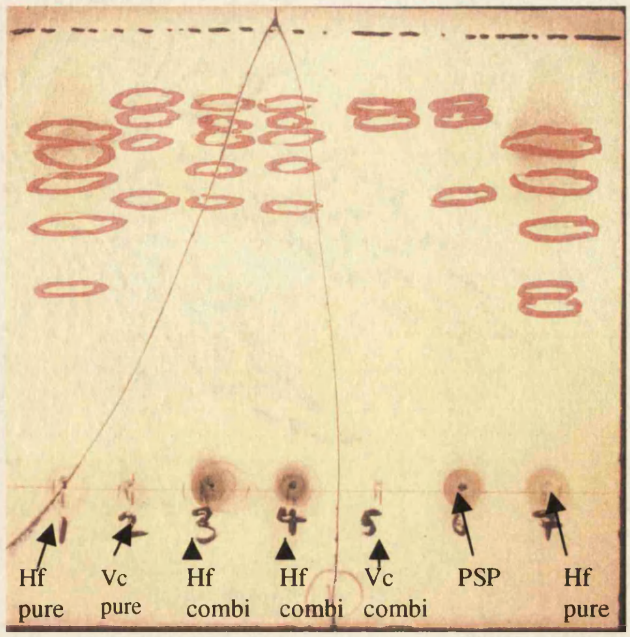
V. commedens gained two high Rf value antioxidant bands in its interactive state, and lost 4 yellow bands visible at UV 366nm. When comparing interactive *H. fuscum* with its young, pure state, the sample lost 2 bands of antioxidant but retained one of lowest Rf value, and gained two yellow and one blue band visible at UV 366nm. The interactive state of *H. fuscum* also lost 2 bands visible at UV 254 nm. However, in comparing interactive *H. fuscum* with its old aged pure culture, the interactive culture lost six UV 366 nm visible bands including the only blue autofluorescent compound produced in any of the samples. This old culture also possesses no antioxidant bands.

6.4.5.2.3.1 TLC age-related trends

V. commedens gains antioxidant bands and one yellow UV 366nm and one UV 254 nm band in the older culture. The older culture of *H. fuscum* lost all antioxidants and UV 254 nm visible bands but gained 10 bands including a blue autofluorescent band mentioned above.

6.4.5.2.3.2 Final TLC plate result

Figure 6.8 shows a second large TLC plate sprayed with anisaldehyde reagent. The PSP zone can be seen to be composed of bands, which are of the same Rf value as those in *V. commedens* and *H. fuscum* interactive states. Interactive samples of *V. commedens* have lost and also gained a band, whereas *H. fuscum* interactive state has gained 2 UV bands and one blue coumarin band, which is unique to all samples. Trends with age show *V. commedens* gaining a UV band but *H. fuscum* loosing 2 bands. What is interesting about the global



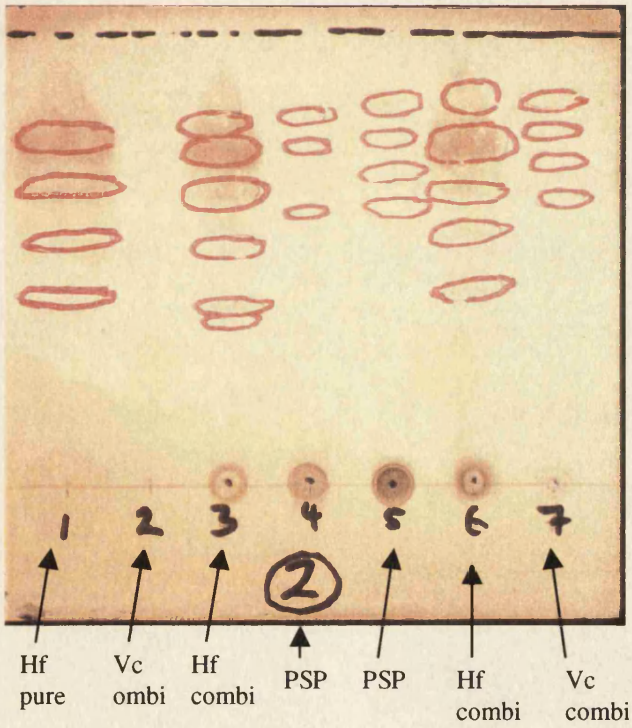


Figure 6.6 Two small thick layer plate, glass-backed chromatographs which have separated out secondary metabolites from both *H. fuscum* and *V. commedens* into bands which have been labelled with the fungal identity and developmental stage from which mycelial plugs were taken. The red circles designate bands visible under ultra violet light - which indicate a variety of coumarin, terpene and aromatic phenolic compounds are present in both fungi.

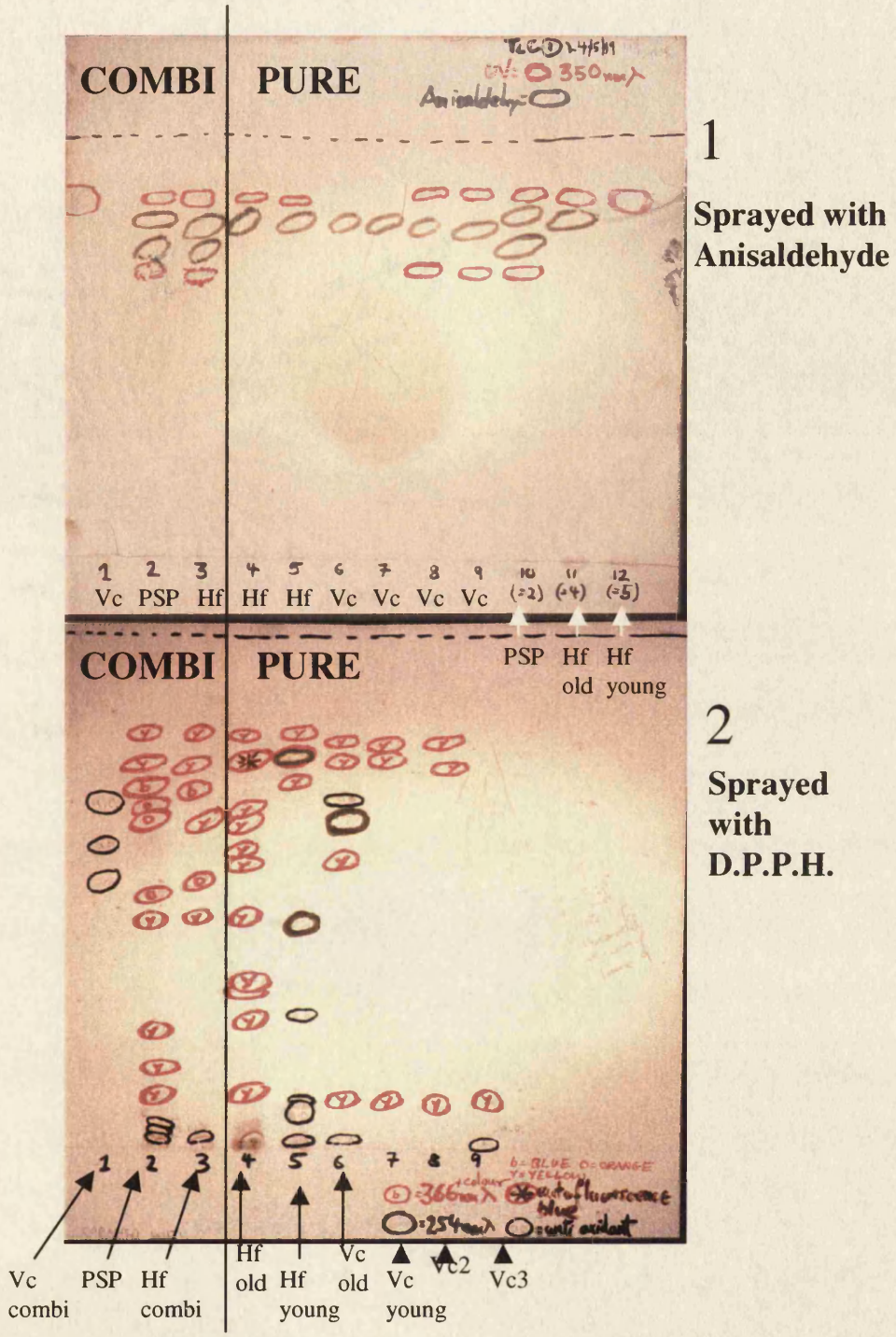


Figure 6.7 Two large thin layer plate chromatographs which separate the secondary compounds of *H. fuscum* and *V. commedens*. After running the compounds through the matrix, the top plate (1) was sprayed with anisaldehyde and observed under UV light. The bottom plate (2) was sprayed with DPPH before observing under UV light. On the top chromatograph (1), dark green bands indicate metabolites which became visible on reaction with anisaldehyde under normal white light. Red bands represent positions of metabolites observable only under UV light of wavelength 350 nm. On the bottom chromatograph (2), metabolites reacting directly with DPPH, observable as bands without UV light, are shown as thin black bands. Positions and colours of metabolites observable under UV light of wavelength 366 nm are shown in red, whereas positions of metabolites observable only under UV light of wavelength 254 nm are shown in thick green. The thin black vertical line separates combination cultures (at left) from pure cultures (at right).

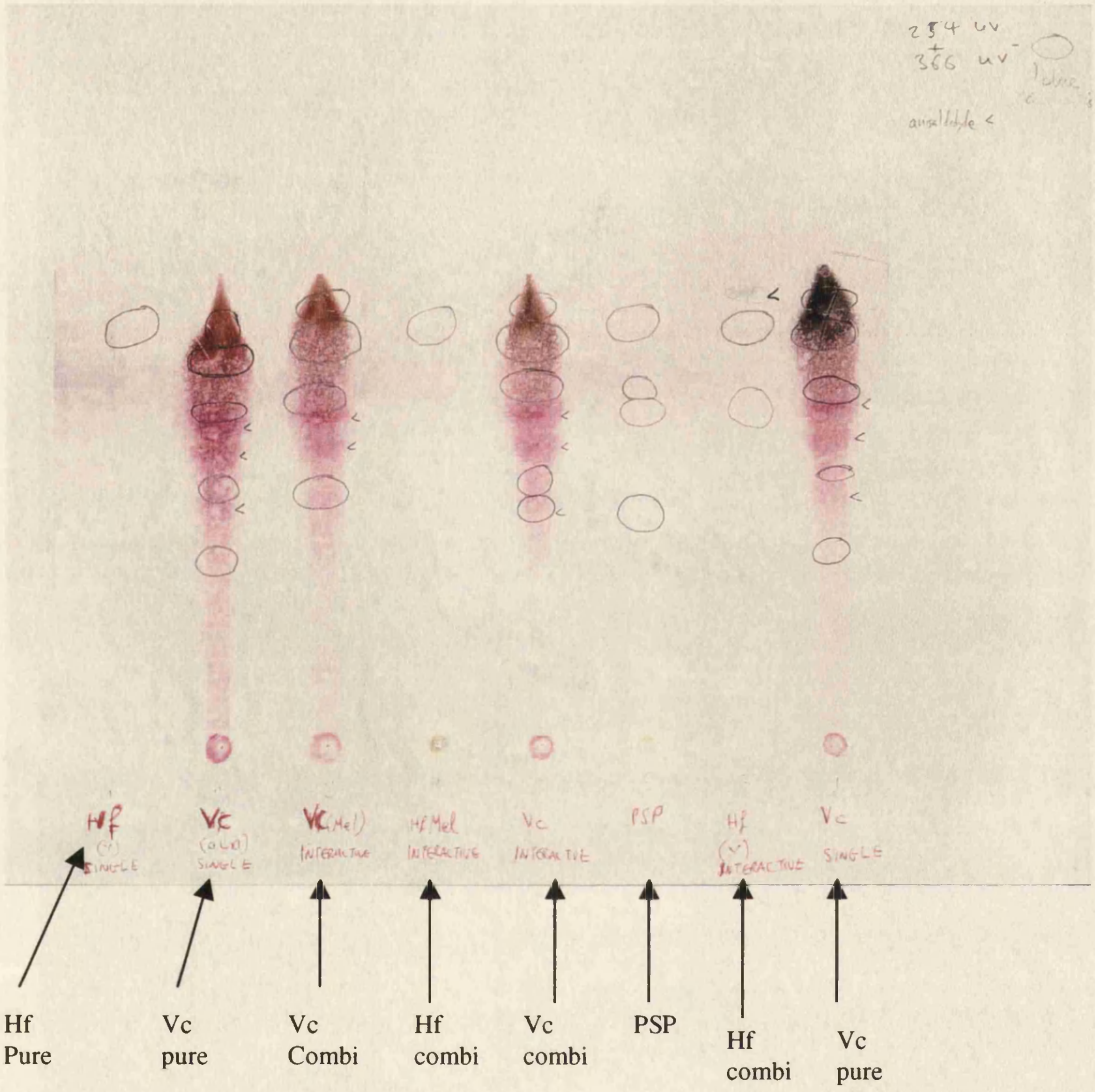


Figure 6.8 Thin layer plate chromatograph showing the separation of chemicals from different states of combined and pure cultures of *H. fuscum* and *V. commedens*.
The colours were produced by reactions with the spray Anisaldehyde; which forms pigments with a variety of complex aromatic phenolic compounds. Circles outline fluorescent bands only visible under Ultra-Violet light, < signs indicate positions of blue staining from anisaldehyde which indicates coumarin molecules. The 8 lanes are labelled with the identity of the fungus and the state of development.

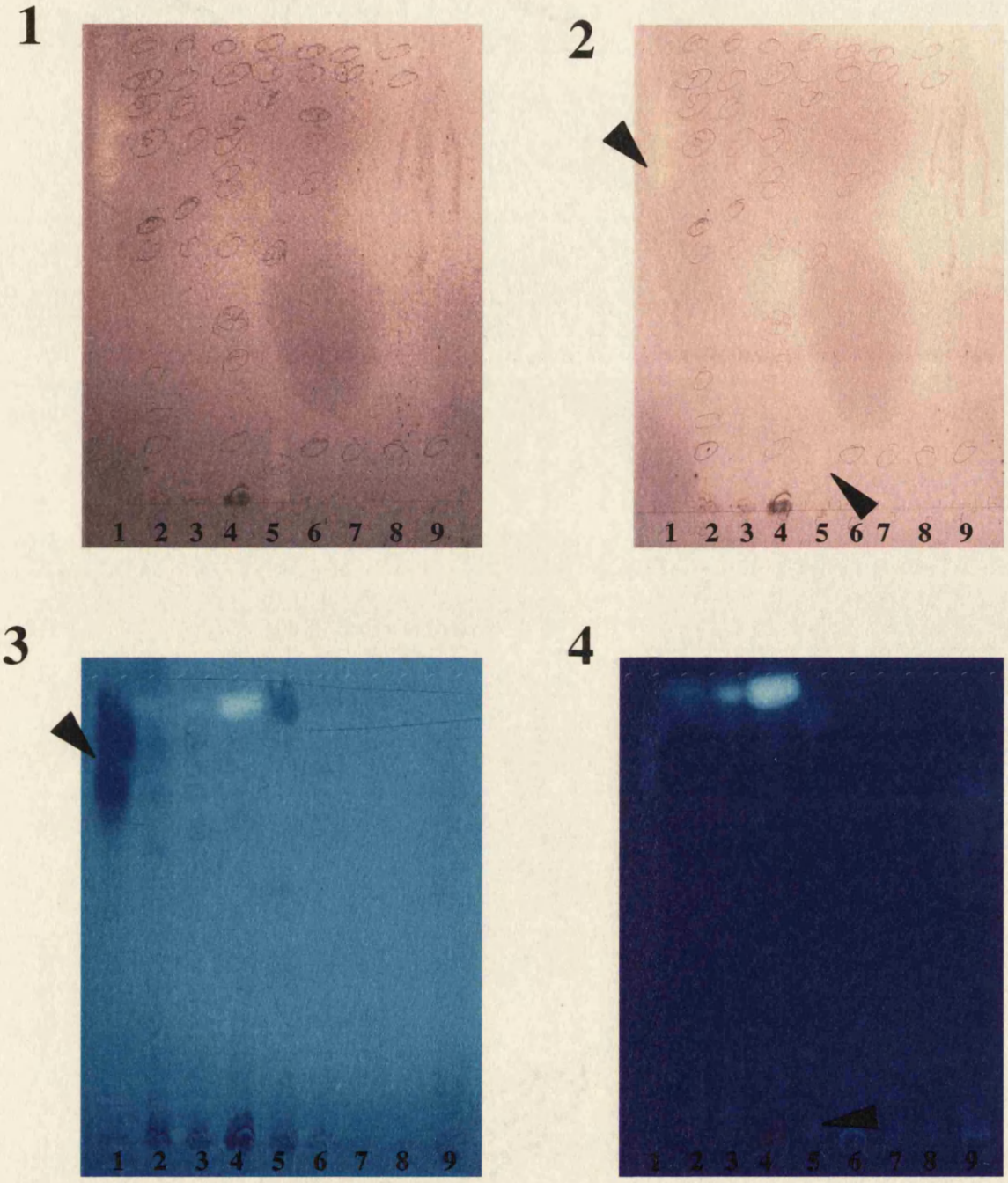


Figure 6.9 TLC separation and detection of anti-oxidant metabolites from pure and interactive cultures of *Hypoxylon fuscum*. Lanes 1 and 2 show bands of metabolites separated from pure cultures of *Hypoxylon fuscum* and *Vuilleminia commedens* respectively. Lanes 3 and 4 show metabolites from interactive *Hypoxylon fuscum* and *Vuilleminia commedens* respectively. Lanes 5, 6 and 7 show metabolites from different parts of the pseudo sclerotial plate zone. Lanes 8 and 9 show metabolites from aged pure cultures of *Vuilleminia commedens* and *Hypoxylon fuscum* respectively. Panels (**1**) and (**2**) show TLC plate as sprayed with anti-oxidant detecting solution DPPH after 2 and 15 minutes respectively. Here anti-oxidants cause zones of clearance. Panels (**3**) and (**4**) show the plate as viewed under 2 wavelengths of UV light. Compounds exhibiting anti-oxidant activity are indicated by arrows.

trend in this TLC plate is that the purple coloured anisaldehyde bands common to all *V. commedens* cultures are absent from PSP and all *H. fuscum* samples. Figure 6.9 shows a repeat large TLC plate sprayed with DPPH to detect antioxidants, under white light (1 & 2) and 2 UV wavelengths, 254 nm and 366 nm, of illumination (3 at 254 nm and 4 at 366 nm). Considering photos 1 and 2, pure *H. fuscum* culture exhibits many antioxidants of high Rf value whereas in the interactive state, antioxidants of low Rf value become visible. Some antioxidant compounds of low Rf value are also active in this *V. commedens* sample. The highest amount of antioxidant activity occurred in the PSP zone sample 5, but there was considerable variation shown by low antioxidant banding in PSP samples 6 and 7. Regarding age, old (i.e. older than 1 month - see chapter 4) culture samples of pure *H. fuscum* lost their antioxidant activity whereas *V. commedens* samples showed some clearing despite the fact that the sample had run off line in a branching pattern (lane 9).

Grouping all bands together to look for trends, interactive *H. fuscum* gained 6 low Rf bands compared to the pure culture. These bands had similar Rf values to those that were present in *V. commedens*. Interactive *V. commedens* lost 1 but gained 3 bands. With age, pure *H. fuscum* lost antioxidants but gained 3 bands with similar Rf values to those present in *V. commedens*. With age, *V. commedens* lost 6 UV bands and gained antioxidant bands.

The global trends for antioxidants were for the potential to find a large increase of bands in PSP zones. Global trends in the UV 254 nm spectra were for high Rf value bands to be unique to pure *H. fuscum* and interactive *V. commedens*. Trends in UV 266 nm spectra were for the most numerous and brightest bands to be present in interactive *V. commedens*, with less in interactive *H. fuscum*, and less again in pure *V. commedens* and the PSP zones. Table 6.2 summarises all TLC results from section 6.4.5.2.

TABLE 6.2 Thin layer chromatography results summary

Key: **++++** = relative amount of banding, ↑ = increased banding, ↓ = decreased banding, — = lack of banding, **polar** = bands found at bottom, **unpol** = non-polar bands found at top, **@ 366** = a wavelength-specific observation, **autflor** = autofluoresncent band, **Yellow**, a band which fluoreses yellow under UV light, **unique** = a compound specific to this mycelium, **no effect** = no change in banding due to interactivity.

STATUS	Monoculture	Mononoculture		Interactive	Interactive	Interactive
Species	*H. fuscuum*	*V. commedens*		*H. fuscum*	PSP	*V. commedens*
POLARITY	polar ++++ unpol +	polar + unpol ++++		polar ++++ unpol ++ @366 ↑↓	polar ++ unpol ++++ unique ↑	polar ++ unpol +++ @ 366 ↓
ANTI-OX	polar + unpol –	polar + unpol +		polar + unpol – @ 350 ↑↑	polar +++++ unpol +++++ 13 bands ++++++++++++	polar ++ unpol ++++ @ 350 ↑
ANISAL	–	unpol +++		unpol ↑ unique ↑	–	unpol ↓
Age- effect ANISAL	↑↑↑	↓↓		@ 254 ↓↓	no effect	@ 366 ↓↓↓↓ (yellow)
Age-effect ANTI-OX	↓↓	↑↑		unique coumarin ↑ autflor ↓↓	no effect	

Regarding the TLC results, the most important effects regarding cecid-fungal relations were that PSP zones had 13 bands of antioxidant activity of highly variable polarity. This suggests that both polar and non-polar antioxidant metabolites were a key feature of the PSP zone. Secondly, the effect of the different developing reagent sprays changed the UV banding pattern in a non-constitutive, reactive fashion.

6.4.5.6 HPLC results

6.4.5.6.1 Interactive signature peaks

Figures 6.10 to 6.13 show HPLC spectra comparisons between pure and interactive and also between young and old *H. fuscum* and *V. commedens* cultures. **The global trend, and most interesting result is that a triple peak sequence observed between 15 and 16 minutes at both 210 and 280 nm UV provides a signature of interaction and PSP zone.** This triple peak sequence interactivity-signature is not found in pure cultures of *H. fuscum* or *V. commedens* at either UV wavelength. The triple peak is strongest in the PSP zone and found to a lesser extent in the mycelial samples on either side (figure 6.12 and 6.13). Aside from this result, there are a number of other peaks to draw attention to. In figure 6.10, a series of peaks only visible at 280 nm UV appears just before the elution of the interactivity-signature triple peak sequence just described. This range is also visible in both interactive cultures but less in *V. comedens* than in *H. fuscum*. However, this pre-interactivity-signature range is not apparent in the PSP zone at either wavelength (see figures, 6.12 and 6.13). **A single peak at 12.6 minutes appears in interactive *H. fuscum* at UV 210 nm, which is not apparent in pure culture (figure 6.10).** Two large single peaks at 8.5 and 17.5 minutes are almost lost from interactive *H. fuscum* at both wavelengths.

6.4.5.6.2 Effect of age on metabolites

Figure 6.11 considers ageing and its effects on *H. fuscum* metabolites. The bottom profile shows the loss, with ageing, of a range of polar metabolite peaks between 5 and 10 minutes at UV 210 nm. and drastic reductions of peaks at 17 and 17.5 minutes from *H. fuscum*. The top graph shows less loss of polar metabolites and the addition of several when ageing is combined with interaction. Here peaks are gained at the triple signature described above (15-16 minute) and also at 10.5, 12.5, 13.5, 14.5, and 20 minutes.

6.4.5.6.3 Interactive with pure culture comparisons

Figure 6.12 shows comparisons which indicate the effect of interaction on the metabolites produced by *V. commedens*. At top right, by becoming interactive (blue line), *V. commedens* loses or severely curtails availability of polar metabolites below 13 minutes in elution time. However after 13 minutes, at the more non-polar end of the metabolite profile, a range of peaks is gained at 14 minutes, and the triple peak interactivity-signature occurs between 15 and 16 minutes. The second isolate of *V. commedens* (green line) shows two unique peaks for *V. commedens* at 6.5 minutes and 16.5 minutes. These are at the very extremes of polarity and non-polarity for this species. Also figure 6.12 shows the PSP as clearly having the most diverse metabolite profile, followed by interactive *H. fusum* and then interactive *V. commedens*.

6.4.5.6.4 Effect of age on PSP

Figure 6.13 shows the effect of age on PSP and *V. commedens* metabolites. The top profiles show an increased non-polarity of PSP zone as it ages, and a large and extensive suppression of the appearance of polar metabolites up to 20 minutes in elution time. After 20 minutes, new peaks are produced at 26 and 26.5 minutes. The middle profiles show the global shift in metabolites between pure, young *H. fuscum* with many polar compounds, mostly produced between 5 and 13 minutes, but few non-polar ones. This is contrasted to young PSP zone, where there is a shift to the right at the polar end with most elution between 9 and 17 minutes. The lower profiles show the effect of age on *V. commedens*. Note the absence of triple peak

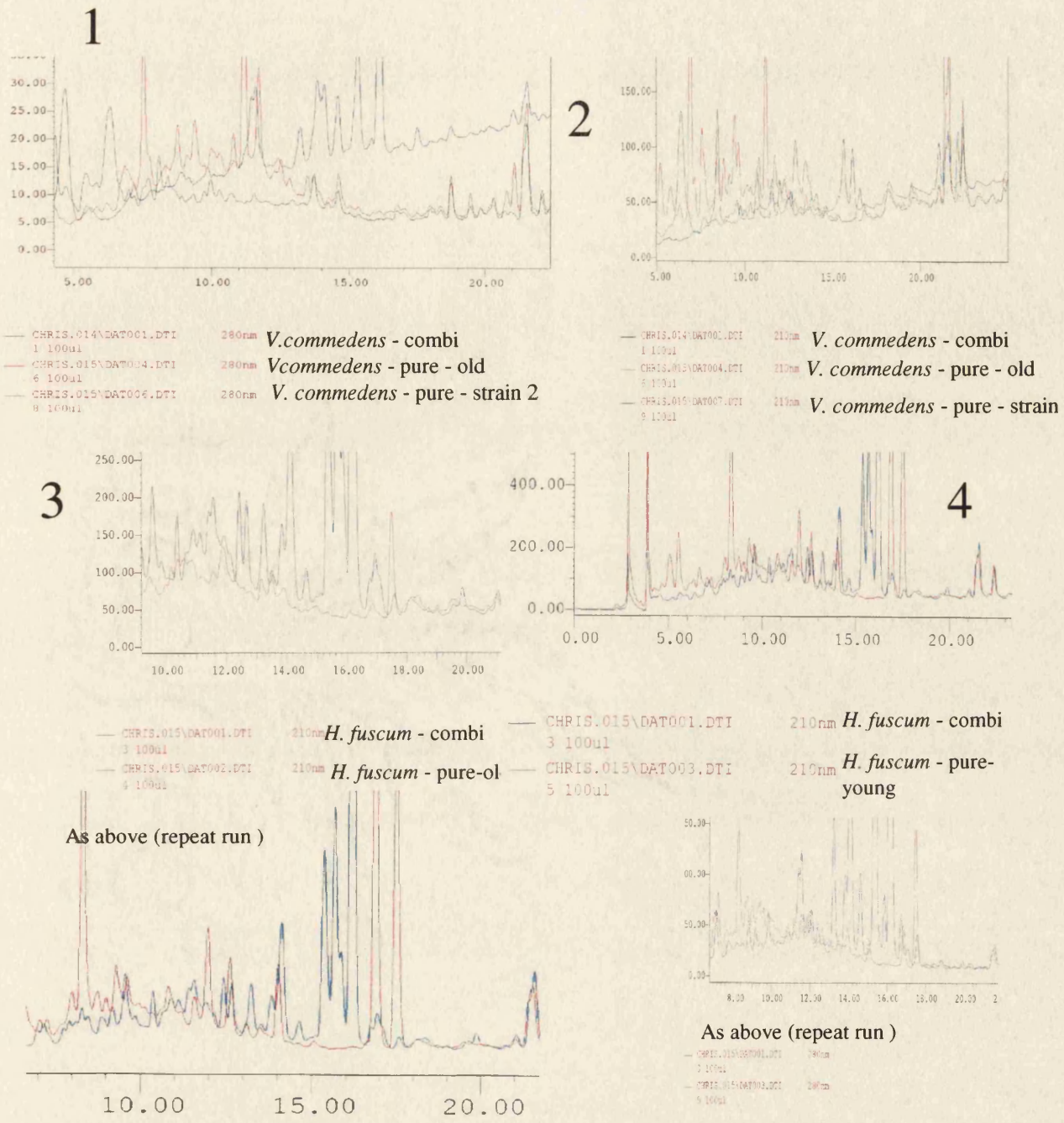


Figure 6.10 HPLC metabolite profile comparisons between interactive and monoculture states of fungi. (1) shows pure isolate 2 *V. commedens*, pure vs. old, and interactive, as does repeat run shown as (2). (3) shows interactive *H. fuscum* with pure and old *H. fuscum*. (4) shows interactive *H. fuscum* with it's pure vs. young forms. With this first run, 10 samples were loaded into the HPLC column.

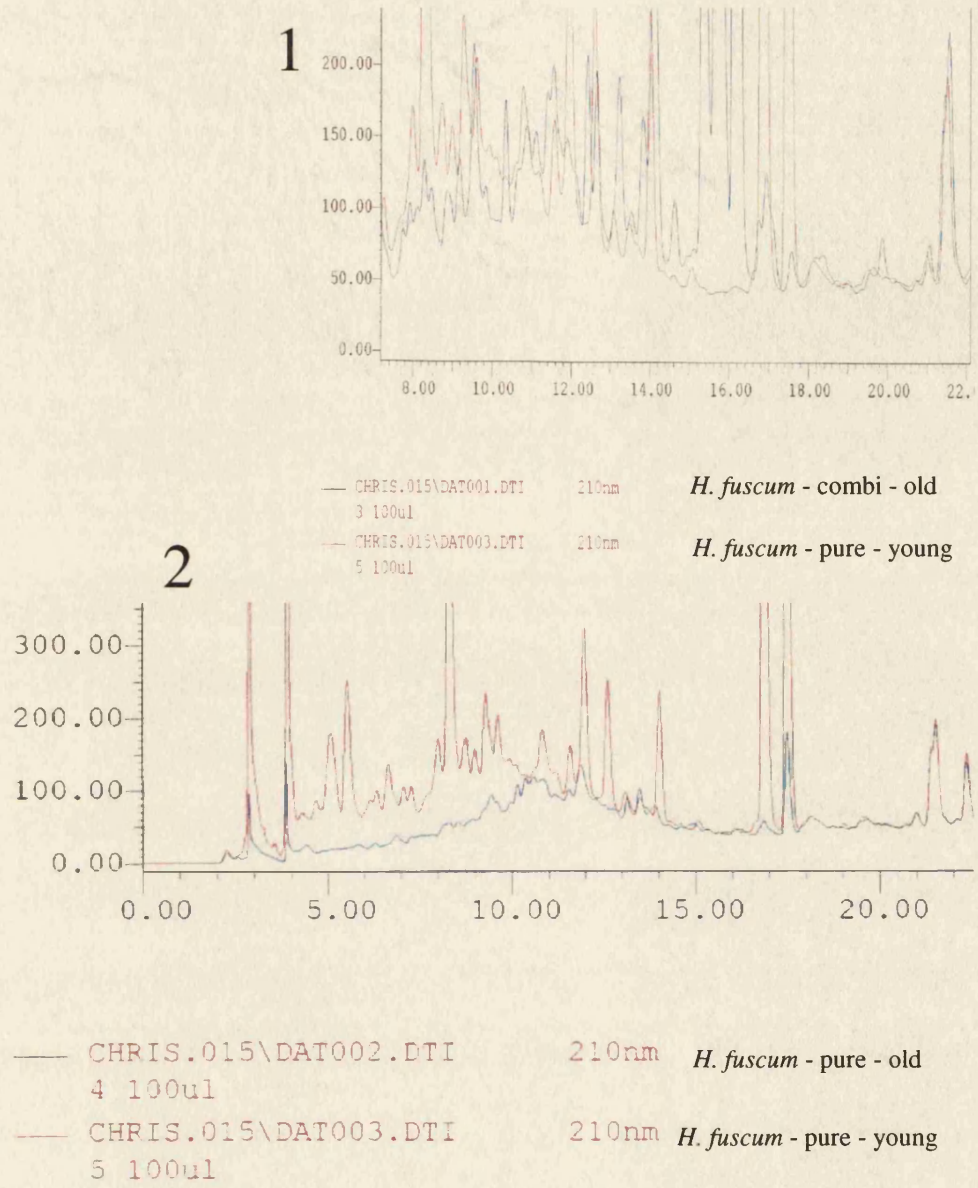


Figure 6.11 Changes with age; HPLC metabolite comparisons between *H. fuscum* at different ages and states of interactivity. (1) shows interactive old (blue line) with young and mono-culture (red line). (2) shows monoculture old (blue line) with monoculture young (red line). 4 runs are exhibited here, in solvent gradient 1 with each profile being one individual run through the HPLC column.

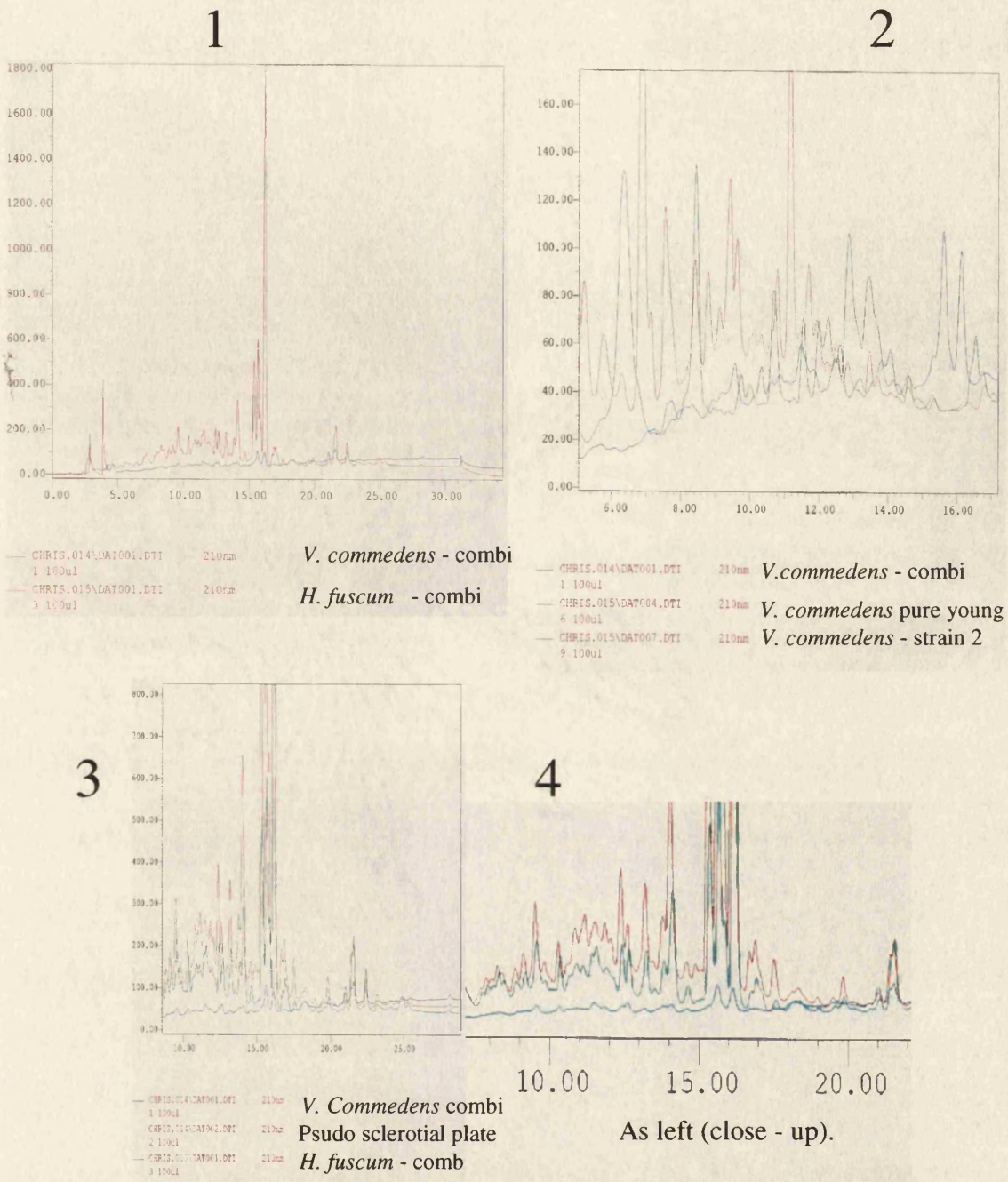


Figure 6.12 HPLC profiles showing solubility of fungal metabolites along x-axis in un-polar highly flammable solvent, towards right, in polar phosphate buffer with Milli Q. water. At top left (1) are interactive *V. commedens* vs. *H. fuscum*. (2) shows pure vs. interactive cultured *V. commedens*. (3) shows interactive metabolites from *V. commedens*, PSP (pseudosclerotial plate) zone and interactive *H. fuscum* as compared in the same run.

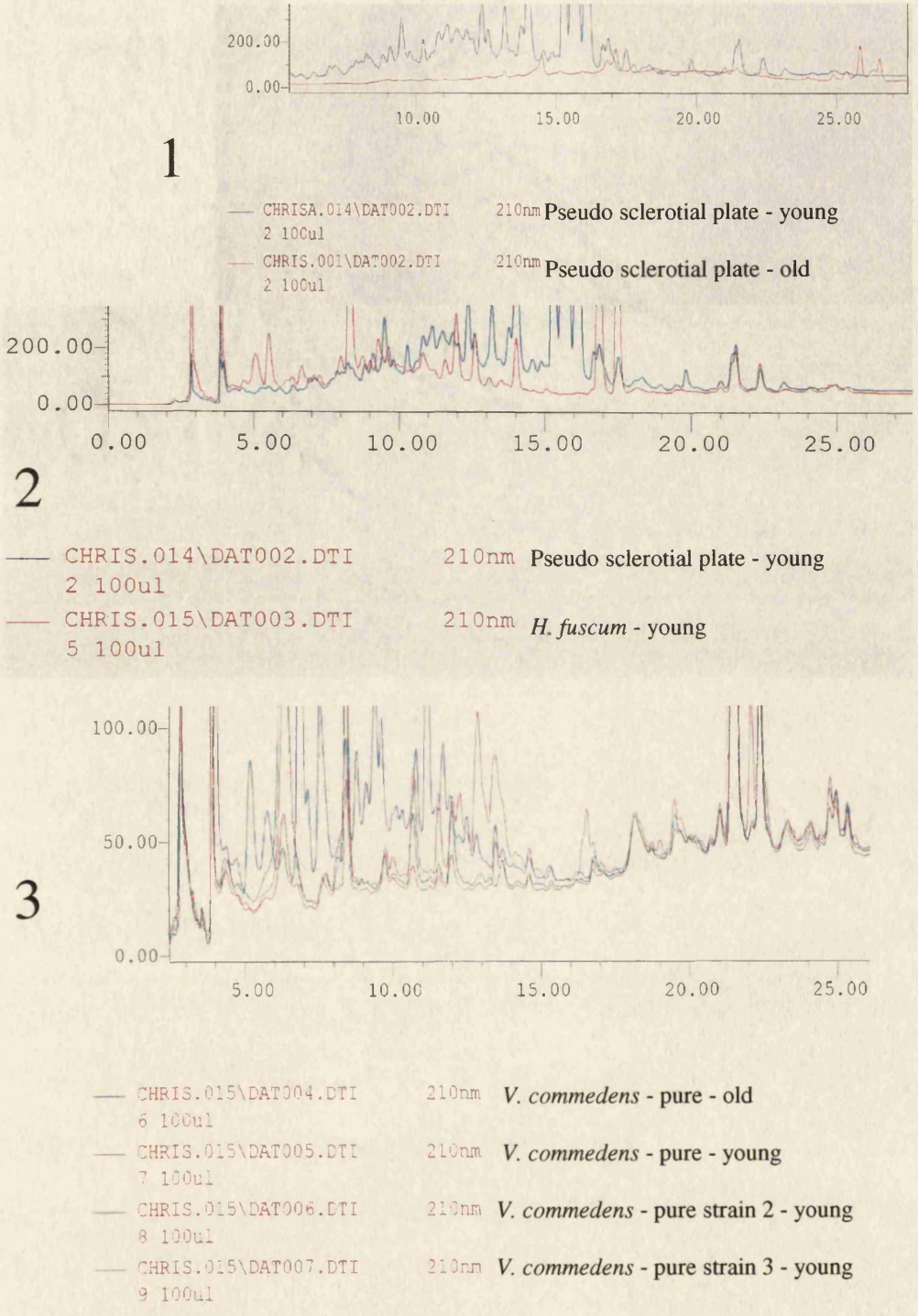


Figure 6.13 HPLC metabolite comparisons between (1); P.S.P. at different ages, (2) *H. fuscum* vs. PSP. (3) shows comparisons between four different pure cultures of *V. commedens,* including isolate 2. Eight samples were run using solvent gradient system 1.

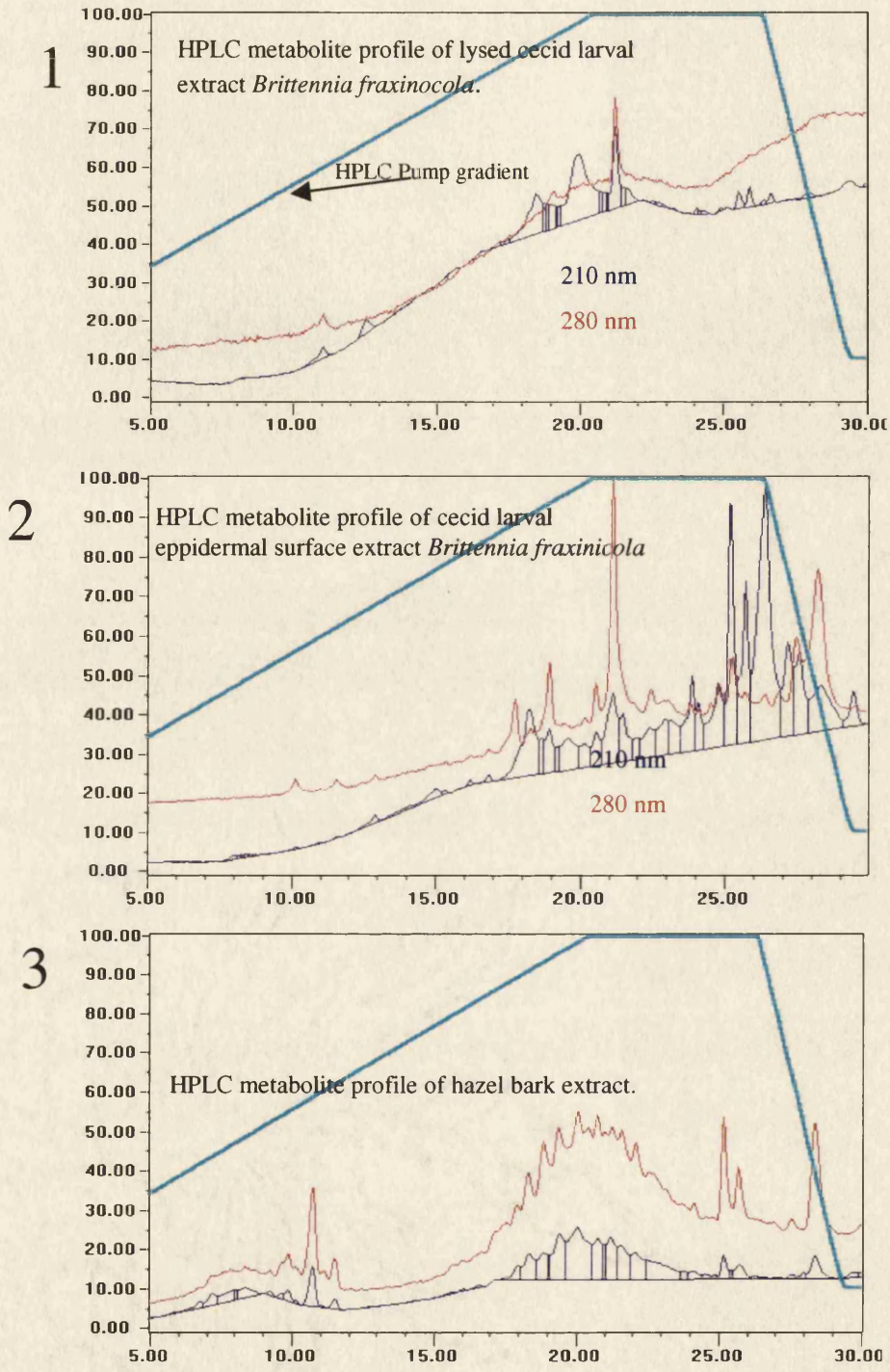


Figure 6.14 HPLC dual wavelength metabolite comparisons; (1) shows metabolites of a lysed cecid extract. (2) shows cecid epidermis wash-extract and (3) shows metabolite extrct from hazel (*Corylus avellana*) bark.

signature between 15 and 16 minutes described above. The variation in pure *V. commedens* occurs at the polar end between 5 and 13 minutes. The older cultures produce more in this range of peaks than the young, pure *V. commedens* cultures. One unique peak (red) stands out at the non-polar end, eluted at 22 minutes, belonging to old *V. commedens* strain 2.

6.4.5.6.5 *B. fraxinicola and Corylus avellana metabolites*

Figure 6.14 shows *B. fraxinicola* cecid larval HPLC profiles and one for hazel bark. The cuticles of these larvae have more metabolites that eluted under these conditions than internal larval haemolymph. A short range of peaks from cecid cuticle's metabolite profile at the non-polar end (27 - 29 minutes) did not occur with any of the fungal extracts, but were also found in the bark metabolite profile peaks at 26 minutes. Cecid and bark profiles had non-polar peak similarity to that of old PSP (figure 6.13). The range of peaks between 18 and 23 minutes were in common with interactive *H. fuscum* (figure 6.10), and the range of peaks between 10.5 and 12 minutes were in common with pure *V. commedens* (figure 6.10).

6.4.5.6.6 *HPLC results summary*

Results for all HPLC comparisons between pure and interactive cultures are shown below in table 6.3.

TABLE 6.3 HPLC Metabolite comparison summary

Key: – = absence of peaks or age effect, + = presence of peaks or age-effect, + + + = relative abundance of peaks, **signature** = triple interactivity-signature sequence @ 210 and 280 nm UV, 15-16 mins, **pre-sig** = a highly variable range of peaks at UV 280nm that occurred in advance of signature, **specific** = particular characteristics, **age effect** = change with older cultures, **@ 210, 5.2, 6.0, 9.5** = at wavelength of UV 210 nm, a range of 3 peaks at 5.2, 6.0 and 9.5 minutes.

status	**Monoculture**	**Monoculture**		**Interactive**	**Interactive**	**Interactive**
species	*H. fuscum*	*V. commedens*		*H. fuscum*	PSP	*V. commedens*
signature	–	–		+ +	+ + + +	+
pre-sig	–	–		+ + +	–	+
specific	– @210-12.5 min + + @210+ 280-8.5, 17.5	+ + @ 210-6.0,8.9		+ @ 210-12.6 – –@210+280-8.5,17. 2	+ + + + @210 + + + + @280 **most abundant and diverse profile**	– – –@210, <13.0 + + + @210, >13.0
age effect	– – – – of polar	+ + @210-5.2, 6.0, 9.5, 11.5 isolate 2 : – – @210-10.0, 12.0, 13.0, 13.5, 15.5, 22.0,		– – – – of unpolar. + + + of interaction signature peaks **abundant and diverse**	+++++ of unpolar ++++ unique peaks	– unique peak@210-22 **fewest peaks of any profile**

Cecid metabolite HPLC results from larvae grown on interactive cultures shared many peaks in common between the two fungal species and also the PSP zone, where a peak at 21 minutes coincides with peaks from interactive fungal and PSP zones. More abundance and diversity of metabolites were found on the larval cuticles than from the lysed haemolymph and lipids, reinforcing the previous suggestion that cecids may act as vectors of metabolites between interactive fungi.

6.5.Discussion

6.5.1 Cecid response to fungal interactivity

The behaviour of cecids in response to biochemical dynamics of interactive fungi showed movements of cecids away from desiccated wells at the edges of pure *H. fuscum* cultures in the repli-plate experiment. This observation suggests that cecids are able to sense their chemical environment via cuticular sensoria and prolific anterior segment sensoria, and sensory palps on either side of their mouthparts. Other repli-plate cecid movements were not enough to be explained by the alteration of mycelial development alone, although there were interesting trends, for example, that all agar wells in the centre 9 high nutrient wells showed a non-increasing or falling population at 80 days whereas populations on agar with low nutrient levels were still increasing at 80 days.

There is good evidence presented here that limits in mother larval lengths attained before giving paedogenic birth varies considerably with changes in environment. Longer larval lengths in combined interactive culture can be attributed to the synergistic effects of interactivity and the presence of PSP zone metabolites such as antioxidants and extra proteins (Salvador 2000, Haken 1980). Results at metabolic scales point to reduction of oxidative stress through increased antioxidant production in PSP zones. The importance of the PSP zone over the possible dietary improvement brought about by foraging in two separate mycelia was highlighted by the finding that no significant difference was measured between larval lengths in split plate combination cultures without interactivity (which lacked PSP zones) but possessing two fungi. An assumption here was that PSP zones were not nutritive to cecids since PSP zones lack hyphae, which cecids need in order to feed. As described in chapter 1, PSP zone hyphae have been sequestered and polymerised into latticed, melanised hydrophobic residues. Also no significant differences in larval lengths were measured between those larvae in different pure cultures, but significantly larger larvae were found in interactive cultures with PSP. **Taken together with the metabolic antioxidant and unique chemistry of the PSP zone, these results strengthen the hypothesis of the antioxidative role of PSP zones in affecting the development of cecid larval lengths. This is because any secondary influences from the constitutive adding of pure mycelia, in terms of extra nutritional benefit, were not enough to explain the significant differences, which did occur with interactivity and PSP present.**

However, emergent larval population sizes became significantly greater on constitutively combined split plates without PSP. This indicates that the number of progeny and time taken for each cecid paedogenic cycle are influenced more by the nutritive influences of mycelia of different species and are perhaps secondarily, and to a lesser extent, influenced by the synergistic interactiveness of mycelial PSP zones (Salvador 2000, Haken 1980). Perhaps the main factor contributing to the larval population size is thus diet diversity in terms of a more complete set of co-factors, vitamins and trace minerals than would be found in pure fungal mono-cultures. This requires further investigation since the nutritive constitution of various combinations of fungal mycelia was not investigated during this project, although work by Hunt suggests that dietary steroids are an important part of cecid growth (Hunt 1997). The nutritional lack of one mycelium may thus be alleviated by the ability to feed in another (Wyatt 2000, Nikolei 1961). What role PSP zones play above and beyond the additive effect of improved dietary provision for developing cecid larvae seems to be to affect the final size mothers become before giving birth, irrespective of life-cycle speed and number of progeny per birth.

These differences in body size and life-cycle results indicate that the environmental factors which determine body size may differ from those which determine life-cycle generation times and progeny numbers. Results of chapter 4 suggested that cecids with a high generation time (Gt) had a small number of offspring (Go) and *vica versa*. Work by Hunt suggested that this could be explained through lipid and sterol influenced metabolism (Hunt 1996), with some sterols found abundantly in fungal mycelia. In the light of chapter 5, where cecids reached larger sizes on interactive cultures with PSP zones, but not on combined and non-interactive cultures which lacked PSP (this chapter), the conclusion reached is that a antioxidant chemical environment leads to the potential for greater body size to be achieved, through reduced oxidative stress. **Larval size seems determined by oxidative stress, whereas population size seems determined by nutrition**. This suggests that for large-sized larvae to go through the cecid life-cycle at faster rates (i.e. have a shorter generation time) and give rise to higher overall cecid population (i.e. have a greater progeny number) (Wyatt 1960), a combination of low oxidative stress and high lipid nutrition must be made available within the foraging ambits of larval trajectories. Thus for body size to be linked to reproductive rate and life-cycle, it is by multiple factors acting in concert - oxidative stress and nutrition. These ideas are supported by previous work by Gabritschevsky (1928), Ulrich (1962) Klingenberg 1997 and Wyatt (1960).

A pattern of conclusions emerge such that interactive cultures with PSP contain larger mothers which grow faster, give birth sooner and have more young than slower growing, smaller mothers which produce less young in pure cultures. A hypothesis emerging here is "That feeding, being the main determinant of body size, is reduced in highly oxidatively stressed environments." Radial margins of cultures possess high amounts of nutrition in the form of active hyphae, but also high amounts of oxidative stress due to the presence of peroxidases and lignin catabolism with consequent release of free radicals. This potential paradox may be overcome by the young cecids' ability to forage very actively, especially first-instars, and over considerable areas, so that they engage with a combination of conditions overall (including PSP). **Foraging to interactive mycelia in order to feed and then back to PSP zones with low oxidative stress would take cecid larvae through consecutive zones of high nutrition with oxidative stress, and then low oxidative stress with low nutrition. In other words there is clearly a trade-off whereby the cecid feeding arena is very nutritive but also very hostile - one in which larvae may have adapted to spend minimum amounts of time, whereas the non hostile PSP refuge has little nutrition.**

Patterns of larval size distribution in relation to fungal oxidative stress levels may have some relation to the thickness of foraging trails. Evidence presented on larval foraging indicates that foraging patterns could be described in terms of fractal properties. This is because path thickness, radii and speeds of larval locomotion all change with scale. Results show the scale of foraging pattern to contract in PSP zones and to expand in pure cultures. The thickness of trails in relation to radii suggest that small larvae create the smallest foraging loops and large mothers create the straighter pathways, which lead eventually to fungal boundaries, where they may give birth. Giving birth in boundary PSP zones, releasing many daughter larvae, shown on the CD ROM film (Appendix 3), perhaps accounts for the significant reduction of mean larval size at PSP zones. Further evidence of this is shown by the finding that loop radii decrease towards PSP.

Furthermore, the foraging loop radii of any particular size of cecid are not fixed but shown to be significantly influenced by the environment. Foraging theory suggests that where foraging loops are tightest and most curved, the organisms are in a favourable environment perhaps with less hostility - for example a refuge site, or an area with greater nutriment. Conversely, in oxidatively stressful environments, where the loops are most expanded, the organisms are actually exploring to find the edges of the unfavourable conditions (Couzin 1999). Following this logic, the loop radius results presented here show PSP zones to be most favourable, followed by *H. fuscum*, and then *V. commedens*. However, there is no mycelium on which to feed at PSP zones, so how can PSP zones be most favourable? The answer seems to lie in the amount of oxidative stress available in each environment. Receptivity to the stimulation by oxidative stress causing metabolites in differing conditions is the simplest way to explain the combination of foraging loop, larval size and velocity results. This suggests that the PSP zones are refuges from oxidative stress, and the interactive domains of mycelia provide less oxidative stress at feeding sites than in pure cultures. Here it may be useful to distinguish between oxidative stress within fungal protoplasm, resulting in production of antioxidants by Ascomycetes in interactive cultures; and oxidative stress within agar or wood due to extra-hyphal catabolism of plant macromolecules such as lignin. It is thought that cecids eat hyphal protoplasm itself rather than the products of fungal catabolism outside hyphae as suggested by Springer (1915). Nevertheless, there could be circumstances where the protoplasm from interactive mycelia is full of antioxidants despite the fact that the inter-hyphal spaces are oxidatively stressful, so presenting a further feeding paradox similar to the one regarding PSP and nutrition above. In this sense, cecids could be protected by the antioxidants in their nutrition whilst also being camouflaged from predation by the oxidatively stressful redox catabolism between hyphae (Stachowicz & Hay 1999).

The larval velocity results show that first-instar larvae move significantly faster than mothers. In addition there is a trend for interactive cultures to exhibit the faster first-instar larval speeds. This means that the smallest foraging loops are also being created with the greatest speed whilst the trails made by larger larvae and mothers in search of a less oxidative stress are made more slowly. This also explains how larval velocity on interactive culture is higher than on pure culture. Foraging loop radii are smallest in interactive culture, due to the presence of less oxidative stress and a more favourable environment in which feeding can take place at a more concentrated and condensed scale. As already discussed, foraging loop formation may follow a fractal distribution, so the smaller loop radii are being made at greater speeds. The space, scale, speed of development and behaviour for mother cecids seems very different to that of the first-instars. In a similar way, for any particular size or developmental state of cecid larvae, changes in relative space, scale and speed of development and behaviour are influenced greatly by environmental chemicals.

6.5.2 Chemical synergy and TLC plates

DPPH is itself a free radical. Anisaldehyde is also a strong redox agent. What the difference between the results of different sprays shows is that, in addition to the effects of producing, for example, antioxidant banding patterns under white light (from the spray DPPH) for which the sprays were intended, the whole pattern of UV-visible banding for the sample has also been changed. This indicates that many previously UV-invisible compounds became UV-visible after reacting with spray reagents. These UV-banding changes were above and beyond the reactions for which these sprays were intended. For example, with DPPH spray, for which this chemical synergy was most apparent, more UV-visible bands, which did not cause clearing and therefore were not free radical scavangers (for which DPPH is used) appeared after the spraying than had

been UV-visible beforehand. This difference between the light-absorbance of bands before and after spraying must have been due to changes in the outer electron orbital states of many of the atoms belonging to UV-invisible fungal metabolites after spraying.

Let is briefly consider the spray DPPH more closely. DPPH is used to detect free radical scavengers. Some reactive oxygen intermediates (ROI) are free radicals (i.e. have an unpaired electron) e.g. $O_2^{\bullet-}$, $OH^{\bullet}$, $HO_2^{\bullet}$. Some ROI are not free radicals such as H_2O_2, (but may later react and produce free radicals). Many ROI scavengers e.g. phenolics (such as those found in non-polar PSP zones), are electron-rich and act by donating an electron thereby neutralising the extra electron which free radicals possess. DPPH acts as a free radical as it has an unpaired electron in its pink colour form. Many fungal metabolites which were sprayed by the DPPH will have accepted its electron (i.e. the metabolites will have been reduced by the free radical). This would also have the affect the UV-absorbance of the metabolite. Such uptake of electrons would explain the synergistic changes in UV profiles observed. Other compounds, for which the DPPH was actually testing, lost their electrons to the free radical (thereby acting as anti-oxidants – by being easily oxidised). Such compounds caused the DPPH to lose its pink colour under white light.

In any sample of metabolites there must be many more compounds that run up the chromatogram than ever become UV-visible. Secondly, above and beyond their intended purpose, these sprays changed many of the abilities of compounds to absorb light frequencies, and hence to react, with important redox effect. The implication of such chemical synergy is that if all these metabolites are within original hyphal protoplasm, the production of one extra chemical, be it an antioxidant or a free radical, can potentially profoundly alter the redox chemistry of the entire metabolic environment within that hypha. That non-target effects of sprays may be interpreted as synergistic with respect to resultant banding patterns suggests that the potential for synergy to occur is enhanced with increasing chemical reactivity between constituent metabolites.

6.5.3 Comparison of TLC with HPLC

High retention fraction (Rf) values were likely to be non-polar whereas low Rf values were likely to be polar molecules since the higher up the TLC chromatograms metabolites ran, the more soluble they were in the non-polar solvent mixture used. For example, those metabolites, such as many polar *H. fuscum* antioxidant bands, which were found at lowest parts of TLC separations, were most likely to be polar i.e. soluble in water (as expected - see chapter 4). HPLC profiles also suggested that *H. fuscum* metabolites are generally more polar than those of *V. commedens*. However, the PSP zone has more than just a mixture between highly polar (more polar than interactive *H. fuscum*) and some non-polar compounds (less than non-polar interactive *V. commedens*). The PSP zone chemistry is unique and thus can also be described as emergent from a synergistic chemical reaction environment. Also this suggests that some highly insulating non-polar compounds are present in PSP with an additional range of polar, soluble compounds with antioxidant properties.

Five DPPH activated bands and 1 ferrichloride activated band were unique to PSP zones, providing further evidence that completely new metabolites are active in PSP zones which are neither in the interactive mycelia on either side of the PSP zone, nor in pure cultures. However, anisaldehyde spray did not reveal a unique PSP banding pattern, suggesting instead that the more non-polar metabolites are constitutively added from those present in interactive *V. commedens* and *H. fuscum*.

Considering the effects of interactivity on *V. commedens*: the number of ferrichloride activated bands decreased, the number of DPPH-activated antioxidant bands increased whilst non-polar UV-visible bands decreased. The numbers of anisaldehyde activated bands were reduced. On the other hand the effects of interactivity on *H. fuscum* were a gain in anisaldehyde activated bands (including the only blue coloured band, indicating the formation of a coumarin (Hahlbrock 1981, Brown 1981) and ferrichloride activated bands, whilst the number of DPPH activated antioxidant bands were reduced and DPPH activated non-polar UV-visible bands were gained. These effects suggest that, as well as new chemicals; there might also be a reciprocal exchange of metabolites from one mycelium into the other. This idea is considerably strengthened by the tissue-printing trends from chapters 4 and 5. **Global trends from tissue printing have also intimated the possibility of a reciprocal exchange of enzymes since interactivity seems to increase catalase, peroxidase and also H_2O_2 production in both fungal species when initially produced in just one of the partners**. The amount of free radicals at colony margins decreased in interactive culture. What emerges is a pattern of results, which demonstrate that interactivity increases in the one partner what the other partner possesses most strongly in pure culture, and *vice versa*. In addition to a possible sharing of enzymes and resultant decrease in overall free radical activity, the PSP zone develops a unique series of compounds, which perhaps may be involved in the sequestering of metabolites and sclerotisation of melanins into the hydrophobic matrix of which pseudosclerotial plate is composed. The ascomycete genus *Hypoxylon* sp. is known for its production of novel green pigments such as hypoxyeylerone (Edwards *et al.* 1991). The secondary biochemistry of ascomycete fungi seems at least as diverse as the dynamic turpenoid metabolism in plants (Raman 1988, Clayton 1970, Raffa & Smalley 1995).

Such suggestions of the synergistic effect of a metabolic and enzymatic reciprocal exchange between interactive mycelia are added further strength by HPLC analysis. The PSP zone and interactive mycelia of both species are shown to possess a distinctive triple peak produced between 15 and 16 minutes with solvent gradient system 1. Furthermore, this triple peak signature seems to diffuse into interactive cultures, for it was found to be strongest in PSP, and variably strong in the interactive mycelia of each species. This suggests that the triple peak is probably not the same series of compounds as that detected from TLC analysis to be specific to PSP. Rather, this intimates that there is a unique series of PSP compounds which are not found anywhere else and cause other secondary compounds to be formed in the PSP zone and to diffuse from there outwards into surrounding mycelial domains.

The fact that PSP zone HPLC profiles generally possessed more antioxidant peaks and less non-polar peaks than other samples suggests that the zone indeed performs as an oxidative-stress-free zone. Also the antioxidant elution times show considerable overlap with those of *H. fuscum*. This suggests that antioxidants from *H. fuscum* diffuse into the PSP zone and greatly influence its overall redox effect and thus its ability to be a refuge from oxidative stress (Hawkins, Matthew & Hockbery 1993). Furthermore, non-polar peaks in *V. commedens* which seem to disappear from PSP zones suggest that these are possibly polyphenolic and terpenoid free radicals or reactive oxygen intermediates, similar to plant tannins, which become sequestered by the antioxidant properties of the PSP zone (Haslam 1981).

It is not known of what significance the DPPH and anisaldehyde activated autofluorescent coumarin found in old cultures of interactive *H. fuscum* might be. Perhaps it is a chemical produced constitutively throughout

older *H. fuscum* mycelium which performs much the same role in sequestering free radicals as does the PSP zone as a whole, or perhaps it is a signature of the presence of free radical stress on the ascomycete system - a chemical which could enable greater amounts of hyphal insulation and the kind one would normally expect to find more of in Basidiomycetes. However, the chemical was not found in *V. commedens* and so is likely to be produced *de novo* in *H. fuscum* as a response to oxidative stress and resultant ageing (Wilson 1992 b).

Results from this chapter suggest that interactions between the ascomycete *Hypoxylon fuscum* and the basidiomycete *Vuilleminia commedens* exhibit the potential for chemical synergy. Synergistic metabolism i.e. in the PSP zone with cecids, seeded from a potential for chemical reactivity from the interactions of the *H. fuscum* and *V. commedens*, are those where properties of combined events are more than what would be expected solely from a constitutive addition of separate components. New banding patterns and HPLC peaks occurred in PSP zones that did not occur anywhere else. This evidence for synergistic chemical emergence stands even without the additional perturbence of cecid larvae or field conditions. The synergy therefore seems a result of initial conditions and dynamics of reactive interface between fungal species that were different enough for their reciprocal exchange of reactive oxygen species to lead to synergistic metabolic shifts, out of which new metabolites and structures became realised (Prigogine & Stengers 1984). It is this process which it appears cecid larvae have evolved the capacity to enhance to their advantage by perturbing this saprotrophic fungal inter-species, between sub-division interaction effect.

In conclusion, the laboratory results deliver evidence for a dynamic relationship in which oxidative stress and nutrition act as key environmental factors to balance in the developmental feedback, which exists between cecids and fungi. Indeed synergistic properties of biochemically reactive interfaces between mycelia allow the emergence of refuge sites from oxidative stress in the form of PSP zones. Effects of cecids on PSP zones create more refuge for their paedogenic reproduction, from which first-instar larvae can forage from one side to the other feeding on reactive mycelia, gaining a balanced diet and mediating the exchange of metabolic reactives from one mycelia to the other, so increasing the thickness of PSP zone in the process. The whole process can be described as developmental feedback (Kulathind and Sing 2000), especially taking on board the work of cecid cytologists who found out about cecid polyploidal mitotic chromosomal elimination (Ulrich 1943, Nikolei 1961). This is more often associated with plant genetics than that of insects. Indeed, cecids are similar to plants in their mode of genetics and reproduction in at least two ways, firstly in possessing high polyploidy and dispersal behaviours which are similar to some types of fungal spore or plant seed dispersal and secondly waiting for passing air currents before launching propagules into them, or sticking to bird feathers and feet (Wyatt 2000). Also the explorative paedogenic relay effect enabling dispersal to new standing hazel poles is similar to plant clonal growth via ramet runners and stolons, for example strawberry plants, dune-slack sedges and dune-building marram grass.

Insect polyploidy and genetic plasticity (Stadler *et al.* 1998) within cecid equivalents to plant ramets may be explained by building a general synthesis involving the field distribution findings from chapter 3. However, before we can do this, a missing component of work needs to be presented: **how do the above dynamics from laboratory cultures operate within rotting wood itself**? This then is the topic of the following chapter 7.

CHAPTER 7: LABORATORY STUDIES OF INTERACTIONS BETWEEN CECIDS AND FUNGI FROM DECOMPOSING HAZEL WOOD, IV: EFFECTS ON PATTERNS OF BARK ATTACHMENT AND DETACHMENT

7.1.Synopsis

The decomposition of wood is examined in detail through a controlled decay experiment on hazel (*Corylus avellana*) wood slices. Initial physical and chemical conditions are investigated and related to the emergent chemical and physical properties of PSP zone sub-cortical architecture with treatments of fungal species and increasing amounts of fungal interactivity of wood slice fungal communities with and without *Brittenia fraxinicola* cecid larvae. Results indicate that physical potential is seeded by chemical potential.

7.2 Introduction

Chapters, 4, 5, and 6 have built up a body of evidence on the emergence of synergistic chemical and some physical properties of pseudosclerotial plate (PSP) zones between interactive interfaces that define the relationship between the ascomycete *Hypoxylon fuscum* and the basidiomycete *Vuilleminia commedens*. The mechanism of feedback between *B. fraxinicola* cecid larvae and the interactive mycelial duplex that produces a PSP zone is likely to be centred on causal networks involving oxidative stress and the shared metabolic ways by which organisms quench its effects. This scenario helps to explain the field observations of chapter 3 from a chemical standpoint. However, results from chapter 3 point to the physical conditions, rather than chemical, which emerge in interactive mycelial domains and PSP zones in rotting wood. Fieldwork from scales 1 to 6 (see chapter 3) has highlighted the importance of an overtly physical potential to explain *B. fraxinicola* larval distribution and, to some extent, their behaviour. Such a view of the system hardly intimates a strong chemical involvement. However, laboratory studies at scale 7 have highlighted the importance and involvement of a chemical potential, which also helps explain larval distribution and behaviour, and intimated a partial physical involvement in terms of the emergent physical properties of interactive cultures. There seems to be a missing component of investigation, which now must be considered before these contrasting perspectives on the *Brittenia-Hypoxylon*-PSP-*Vuilleminia* system can be reconciled. Only then can we hope to build a broader general schema. Here it needs to be remembered that interplays between cecids and fungi occur within dynamic contexts of growing and decomposing wood. How then do the characteristics of wood itself affect interactions between insects and fungi as the arena in which they relate gradually decomposes? This is the topic of chapter 7.

Figure 7.1 shows cross-sections through *B. fraxinicola* cecid colonies inhabiting the cavity matrix of PSP zones between interactive mycelial decay columns. These zones of wood cavitation are particularly apparent at places where bark has softened and lifted and medullary rays have maintained structural integrity whilst inter-medullary lignin has been removed. The work presented in chapter 7 examines how cavitation of wood occurs over time by introducing the factors which influence the decay of hazel (*Corylus avellana*) wood in a controlled experiment, examining this data for strongest relationships and introducing scanning electron microscopy of emergent PSP zones and HPLC analysis of chemical metabolites.

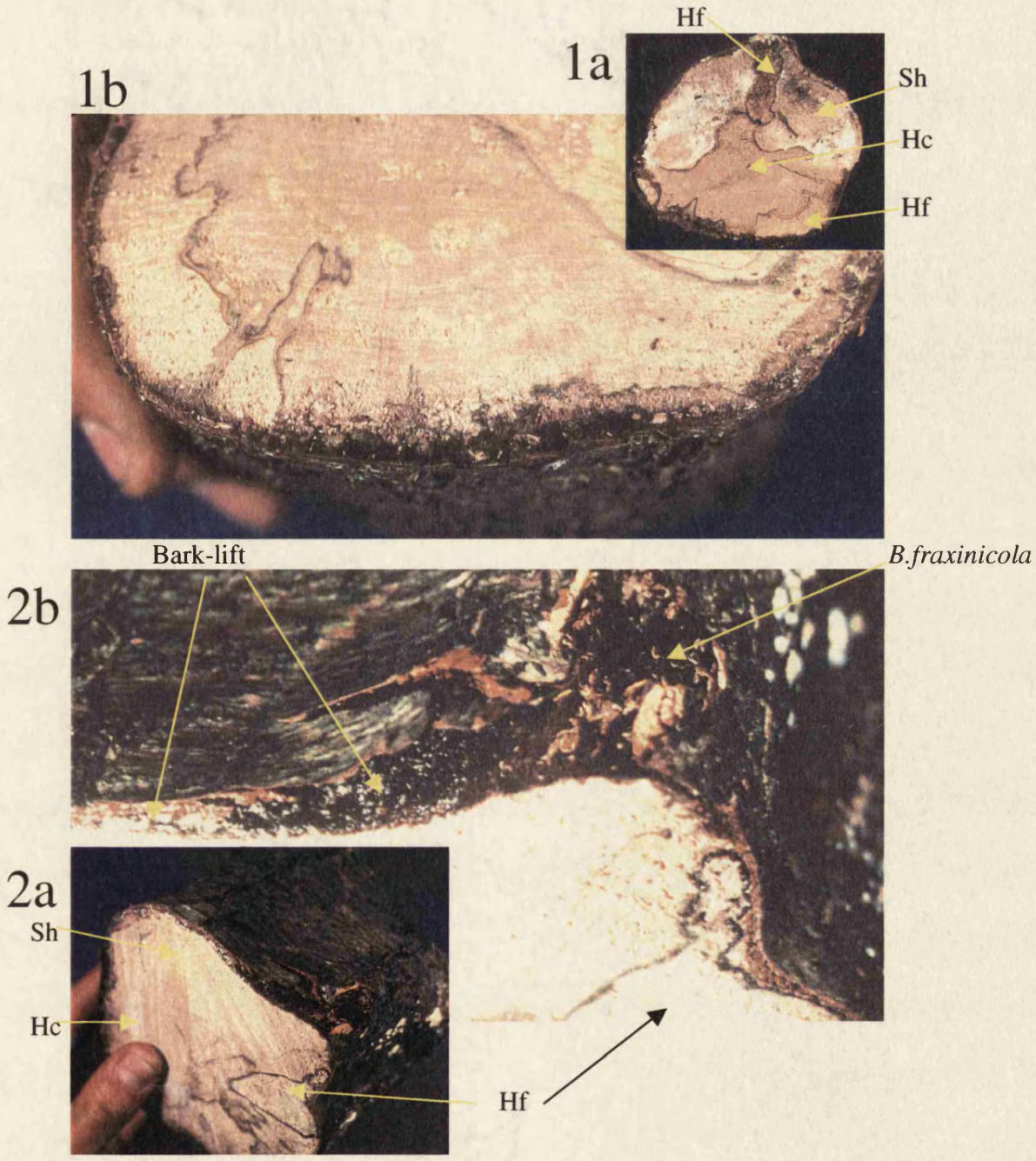


Figure 7.1 Log slices from the field showing PSP zones between *H. fuscum* (Hf), *Hymenochaete corrugata* (Hc)and *Stereum hirsutum* (Sh). (1a) shows a cross-section where interacting basidiomycete and ascomycete species have created deeply pigmented PSP zones, particularly at the bark-sapwood interface. Here bark lift occurs (1b) thus revealing an emergent cavity-matrix fractal architecture between medullary rays. (2a) shows a side view of a similar log slice exposing PSP zones and lifted bark spaces which are heavily PSP-pigmented (2b). *B. fraxinicola* hemipupae can be seen on a PSP zone between the ascomycete *H. fuscum* and basidiomycete *Stereum hirsutum.*

7.3.Methods and materials

7.3.1 Wood slice decay experiment

Three hundred and one wood slices were recorded for initial conditions, treated, arranged in 50 ice-cream containers, left for 10 months, and then recorded for emergent conditions. This wood slice experiment was conducted in ice-cream tubs with ventilation holes and flooded with water. Each wood slice was held above the water surface by a stainless steel rack of pins and pushed into place by inserting pins into pre-drilled holes cut into the wood slices before fungal inoculation (figure 7.2). Fungal inoculation with *H. fuscum* (Hf), *V. commedens* (Vc) or a combination of both had been carried out in Petri dish "sandwiches" from the time of slicing live hazel poles (coppiced from the study site) for two months prior to experimental set-up. Before fungal inoculation, all hazel slices were measured for initial physical dimensions and initial conditions, including bark and wood hardness and thickness. Once fungal mycelium had ramified itself throughout the wood slices, and these had been arranged inside the containers, 10 live *B. fraxinicola* cecid larvae were transferred from standard culture (chapter 4) to the top of each cecid-treated decomposing wood slice. The photos in figure 7.2 were taken 10 months after cecid introduction. All emergent physical and biological characteristics were re-measured at that stage to compare changes between treatments and terminate the experimental time course. This experimental set up can be seen in figure 7.2. (1) shows "control-controls" which were subjected neither to fungal inoculation, nor to cecids, nor to water. They show the wood slices in preserved state with little change between initial and emergent conditions. (2) shows the result of 10-month decomposition with different fungal treatments but no cecid treatment. (3) and (4) show treatments with fungi and cecids combined. Controls had no fungal treatment, but, due to the humidity provided by water-flooding the containers, developed their own fungal communities - primarily from "latent hyphae" which had been quiescent and lying dormant within the hazel pole whilst it was still growing in the study site. Cecids were mostly confined to each wood slice as if on islands. Larvae could only move from slice to slice by "jumping" (chapter 3) as the water prevented other migration. Thirty containers with seven slices (3) and (4) of figure 7.2 had cecids placed on three fungal-treated slices, leaving the other three fungal treatments for colonisation by larval "jumping". In this case controls were also available for the study of cecid colonisation and population development regarding source-sink dynamics. In 20 containers each with four slices – (4) of figure 7.2, three were fungal treatments with a fourth control (for latent fungal development). Ten of the 20 containers remained without cecid treatment whilst 10 replicates were treated with cecids by introducing 10 larvae on to the fungal-treated slices and leaving controls to be colonised by cecid "jumping".

Before the controlled wood-decay experiment was set up as above, wood slices were cut to have the same initial bark area despite variations in the diameter of hazel poles from which they were cut with a band saw. The basis of the decision to keep bark area constant was that it was the bark layers in which the relationships between cecids and fungi are played out, and which were consequently more interesting than the cut surfaces of logs. If bark area is initially constant, then variation in bark at the end of the experiment is more likely to be as a consequence of cecid and fungal interplays.

Two live hazel poles were coppiced from the field site and cut into 320 slices of the same 20 cm^2 bark area by varying the thickness of wood slices according the the diameter of the wood. This was done by constructing a table relating diameter of wood to thickness of slice which would achieve a constant 20 cm^2

bark area (using equation for surface area of a tube = π d t where d is diameter and t is the length of the slice to be cut).

Before any work was done to treat logs in preparation for placing into the ice-cream tub experiment, initial physical conditions of the slices and bark layers were measured. These included bark thickness (mm), inter-medullary ray distance (mm) (measured to the nearest 0.01 mm using a binocular microscope at x 40 magnification with a calibrated eyepiece graticule). Three measurements of each of these were made and the mean average calculated to write down in an initial condition data table. The number of growth rings (age in years) was also counted. Wood and bark thickness were measured using a hardness probe with an end which was 1.2 mm in diameter and the force, measured with a spring on a calibrated scale, measured in N/cm^2. To measure bark hardness the probe was pushed through the three layers of hazel bark until it had just reached the sapwood, whilst the maximum value was read off the scale. For wood hardness, the maximum reading for a full penetration of sapwood to a depth of 1 cm was made. If the initial measurement went off scale, the data written down were scored as >9000 N/cm^2. These measurements then represented initial physical conditions, from which any emergent trends could be established after the 10 months by making the same measurements on the same logs. Individual logs were thus labelled after measuring initial conditions because it was very important to be able to follow each individual wood slice through the entire preparation protocol and ice-cream tub treatments, until re-measurement at the end of the experiment 10 months later. The identities for each wood slice were written on the lids of ice-cream tubs during the decomposition process.

For each type of arrangement of slices within a vented ice-cream tub, control groups were set up. There were two types of control group. The first comprised slices that were exposed neither to water nor treated with fungi or cecid larvae (control-controls shown figure 7.2 - 1). The second type of control comprised slices that were not artificially treated with inoculated fungi but were allowed to develop latent colonisation of fungi (latent fungi), which had been dormant within the living hazel poles before being cut. These latent fungal control slices were also treated with and without cecids, so that within certain comparisons they became part of a fourth treated group (slices treated with latent fungi).

In addition to latent fungal development, three artificial fungal treatments of slices occurred before placing logs into sandwich boxes. This was done by incubating slices sandwiched between two agar plate cultures containing the species to be used: 2 x *H. fuscum* plates, 2 x *V. commedens* or a combination (one culture plate of each fungus). The wood slices to be artificially treated with fungi were placed between agar plates containing standard laboratory fungal cultures (chapter 4) that were then taped together, with wood sandwiched between fungi, and incubated for two months before being placed into ice-cream tubs in the experimental set-ups shown in figure 7.2.

Including the latently colonised slices, there were thus four fungal treatments: combined *V. commedens* / *H. fuscum*, separate *V. commedens* and *H. fuscum*, and finally latent growth. To artificially treat experimental wood slices with fungi, 360 Petri dish cultures of laboratory fungi were required from standard culture protocols described in chapter 4.

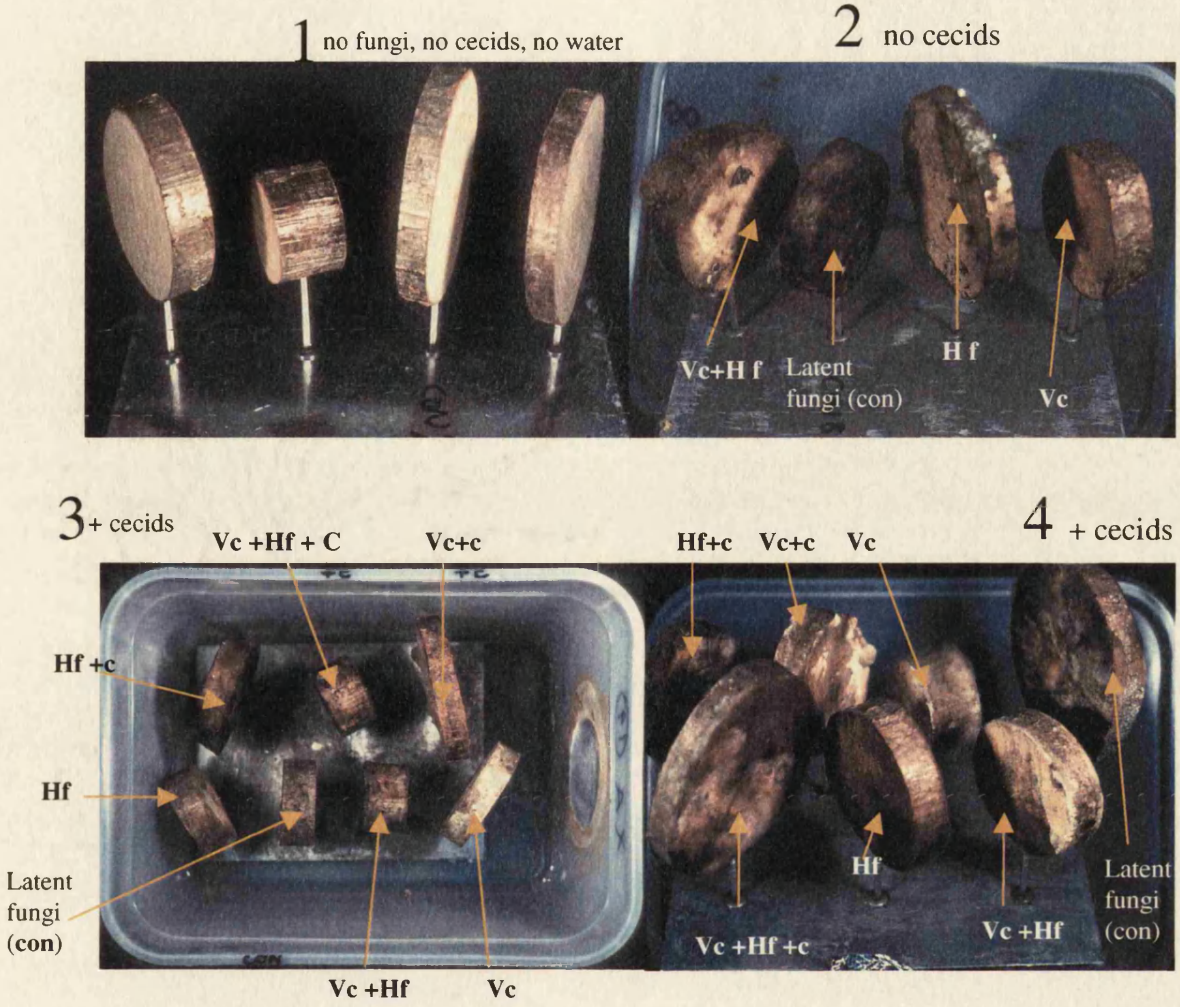


Figure 7.2 (1) shows control-controls which were subjected neither to fungal inoculation, nor to cecids, nor to water. They show the wood slices in preserved state. 2 shows the result of 10 month decomposition with different fungal treatments but no cecids. 3 and 4 show treatments with fungi and cecids. Controls had no fungal treatment, but developed their own fungal communities from latent hyphae within the living hazel pole. Cecids could only move from slice to slice by jumping as the water prevented other migration. 30 containers with 7 slices (3 and 4) had cecids placed on 3 fungal treated slices, leaving the other 3 for colonisation by jumping. In this case controls were also available for colonisation - so testing the effect of latent fungal development on wood decomposition and cecid population development. 20 Containers with 4 slices (2) were treated with water and the 3 fungal treatments with control (latent fungal development). 10 of these were also treated with cecids by introducing 10 cecids on to the fungal-treated slices and leaving controls to be colonised by cecid jumping. In all, 301 wood slices were treated and measured in 50 containers.

These 4 treatments were placed out into two forms of array within the ice-cream tubs. Twenty replicate ice-cream tubs (80 wood slices) of one array (figure 7.2 - 1) consisted of the four fungal treatments either with or without cecid larvae (10 reps of each).

The second array, shown in figure 7.2, (3) and (4), consisted of 20 replicate ice-cream tubs each with 7 slices (140 wood slices), 6 of which were 2 replicates of each of the three artificial fungal treatments. The seventh slice was a control latent fungi slice.

The vented ice-cream tubs were pre-prepared by cutting a window in the front and glueing a larger round piece of the finest (0.25 mm) gardeners' mesh available over it. Ice-cream tubs were sealed with their tight fitting lids and all placed on the same shelves in a Biology faculty outbuilding called the Tropical Products Institute (T.P.I.) hut. This heated building had good shelving for all 40 ice-cream tubs to be maintained under the same conditions of humidity and temperature for 10 months. Every two weeks, the water level inside each ice-cream tub was re-filled to a depth of 4 cm, a depth that took the water level half way between the metal plate and the bottom of the wood slices. The reason for immersing in water was two-fold: firstly to maintain a humid environment suitable for fungal growth, secondly to prevent cecids from moving easily (apart from jumping) from wood slice to wood slice - using the principle that cecids cannot climb through a vertical column of water (Wyatt 1963). After 10 months all wood slices were re-measured to obtain emergent physical properties and compared to the initial conditions. In addition to the physical condition measurements of bark thickness, bark and wood hardness (as above), the presence of cecids was noted and counted around the bark layers and on the underside of stripped bark, numbers of fungal decay columns counted, numbers of wood cavities counted at the sapwood bark interface, relative scores made of pigmentation of fungal decay columns (1 = slight pigmentation, 5 = black), colour of fungal decay columns noted, and number of species of fungal mycelia counted. Measurements were made of length of PSP zone at the sapwood surface (mm), length of *H. fuscum* at the sapwood surface (mm), length of lifted or loose bark at sapwood surface (mm), and maximum height of any regions of lifted bark (mm) from the sapwood surface. Each emergent property was recorded in tables underneath the initial condition data (as above).

7.3.2 Statistical analysis

Data were analysed using correlation analysis and t-tests of grouped data according to controls, and the four fungal treatments. For one set of t-test groupings, those fungal treatments treated without cecids were grouped as "water" (i.e. just water was added into the ice-cream tubs in addition to the treated wood slices) and those with cecids as "cecids". For this test, the control group were control-controls as described above and in figure 7.2. With correlation analysis, those relations that are strong of slope (>0.10) and statistically significant ($P<0.05$) are designated with an asterisk in the correlation tables presented in Appendix 11.

7.3.3 Other methods

7.3.3.1 HPLC analysis of PSP-*Corylus* bark complex and liquorice root

HPLC analysis was performed on wood samples as described in standard protocols of chapter 4, using the same modifications as in chapter 6 for cecid and bark extracts. In addition to wood samples and fungal samples from wood, extracts were made from liquorice root and bark. The reason for this was that both melanised *H. fuscum* and PSP zones emitted a strong smell of liquorice. It was thought that analysis of the

metabolite profile of liquorice might give some clues to the identities of chemicals present in the PSP or interactive *H. fuscum* profiles.

7.3.3.2 Scanning Electron Microscopy of PSP structure in wood

Samples of PSP zone with cecids were cut from logs examined using scanning electron microscopy.

7.4 Results

7.4.1 Wood-decay experiment results

The first result to mention is that, due to latent fungal growth in all the "wet" wood samples, the experimental treatments of wood slices did not determine which fungi emerged to dominate wood slices. There was, however, a significant trend (t-test $P< 0.05$) for *H. fuscum*-treated slices to be more deeply pigmented, for *V. commedens*-treated slices to show less pigmentation and for *H. fuscum* and combined treatments to show more PSP than *V. commedens*-treated slices (t-test $P< 0.05$).

There were no cavities as initial conditions, neither were there PSP zones or any visible fungal domains (fungal genets) present initially. After 10 months, however, all slices were covered in mycelium of many colours and types of pigmentation including PSP zones, and many small cavities had been formed. Bark layer cross-sections were extensively colonised by fungal mycelia. Successful cecid movements from inoculation sites on top of wood slice bark into cavities between layers of fungal colonised bark were clearly observed after removal of bark at the end of the experiment. After 10 months, the bark layers revealed many small cavities along interfaces between sapwood and bark layers, and within the triple bark layer matrix. Examination of these showed *B. fraxinicola* hemi-pupae aligned in parallel with medullary rays in long cavities surrounded by pigmented regions of fungal interactivity (Figure 7.3).

Figure 7.4 shows results of those measurements that demonstrate significant correlations between the number of emergent fungal domains within wood slices and both the length of *H. fuscum* colonisation at the bark sapwood interface and the length of the PSP zone at the interface. The small sample size for this top graph is explained by the fact that few wood slices emerged with fungal domains, which were easily identifiable as being a particular species since there were only mycelia, and no fruiting bodies produced. However, there were a few examples where the fungal domains were definitely *H. fuscum* and matched the observations of *H. fuscum* decay columns from fieldwork. Similarly, although there were many pigmented and interactive zones between emergent members of the wood slice fungal community, there was only a small sample size of wood slices emerging with certain PSP zones and not just deeply pigmented mycelium or pigmented somatic incompatibility interfaces (described in chapter 5).

Lower graphs show, with larger sample sizes, significant increases in pigmentation, bark lift length and bark lift height with cecid-treated as opposed to control (without cecids) i.e. "water"-treated and control-controls. The effect of the 10 months of decomposition process was that significant decreases in bark hardness and wood hardness occurred for all treatments when compared with control-controls, but in this case no significant difference was observed with cecid-treated wood slices.

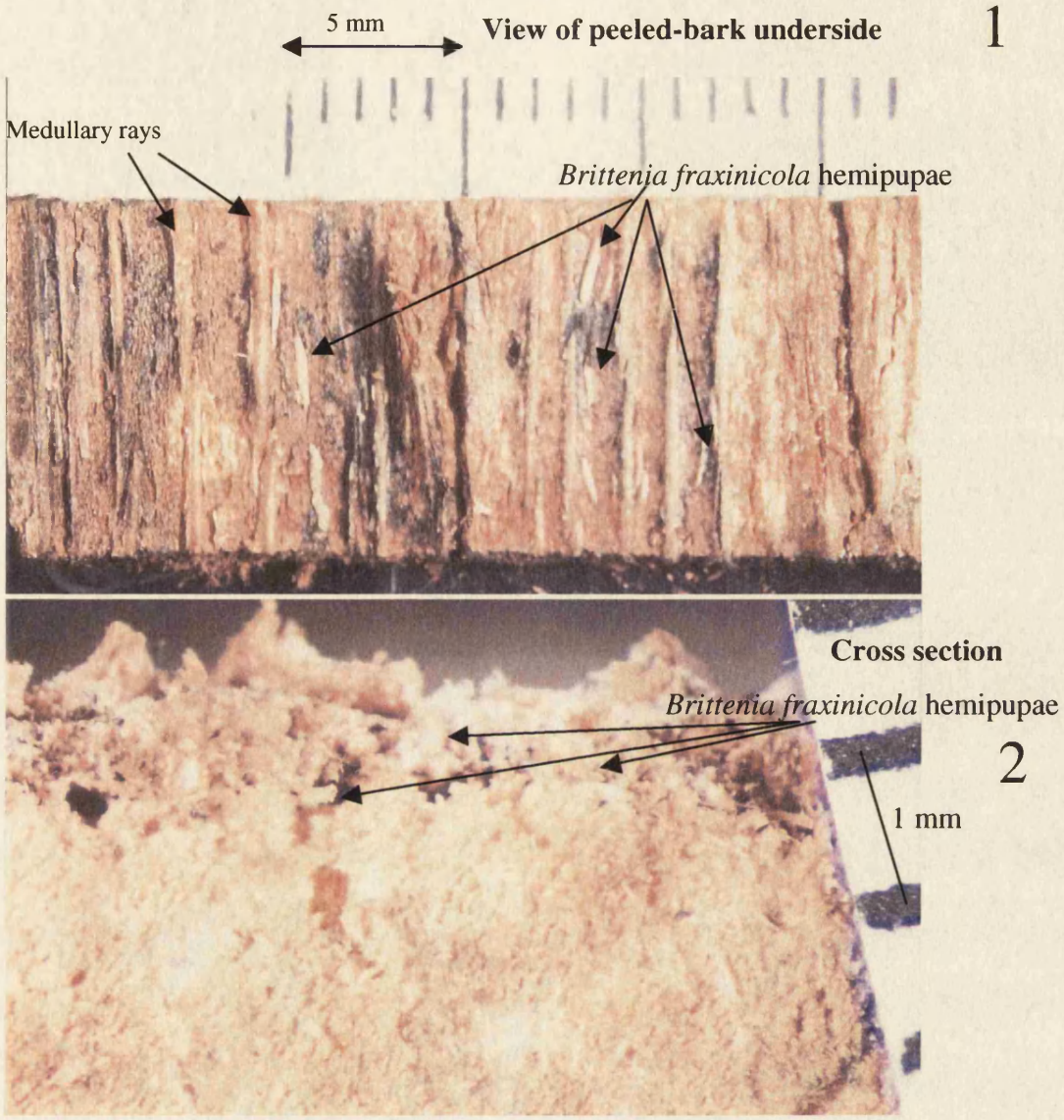


Figure 7.3 (1) shows a view of cecid & fungal- treated wood slices, 12 months after inoculation with *H. fuscum* and *V. commedens* and 10 months after *Brittenia fraxinicola* introduction. *B. fraxinicola* larval hemipupae can be seen lying parallel to the direction of the wood grain and medullary rays in cavities lined in PSP. (2) shows an emergent cavity matrix that can be seen distributed in the sub-cortical zone in cross-section. Hemipupae can be seen end-on within cavities. Divisions are in mm.

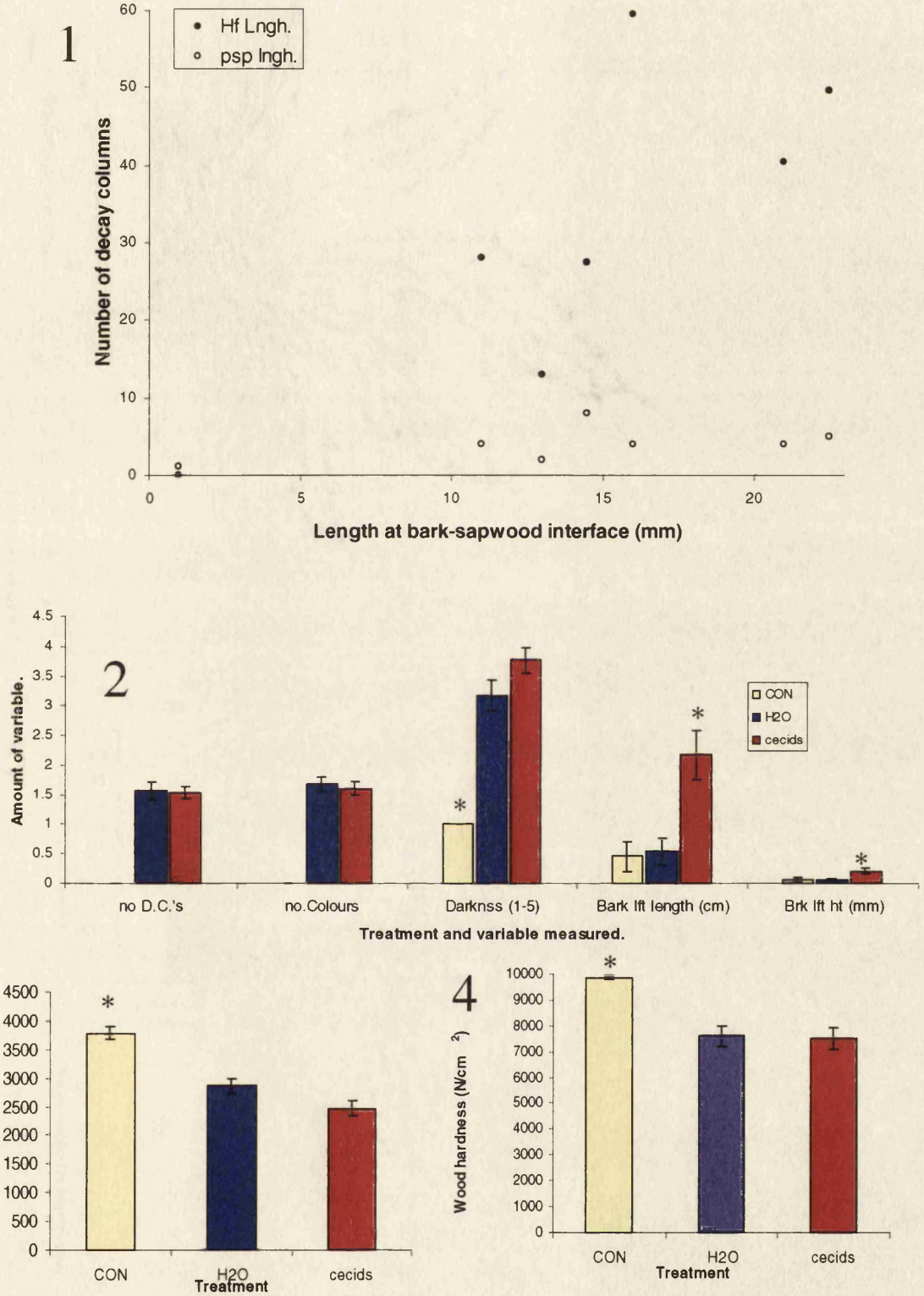


Figure 7.4 (1) shows different rates at which P.S.P. and *H.fuscum* increase within the sub-cortical zone as the number of fungal decay columns rise (n =7, P<0.05). (2) compares variables of logs treated with cecids (n=21, *= P<0.005). (3 and 4) compare effects of water, cecids and controls on bark and wood-slice hardness (n = 21) to see if cecids had an effect on these measurements above and beyond the effect of adding water alone.

Key: D.C.'s =Decay column in wood (bounded by PSP or intra-specific incompatibility zone, no Colours = number of differently coloured mycelia / slice, Darknss = overall pigmentation of wood, bark lft length = length of lifted bark / slice, Bak lft ht = height of lifted bark, * = P<0.05.

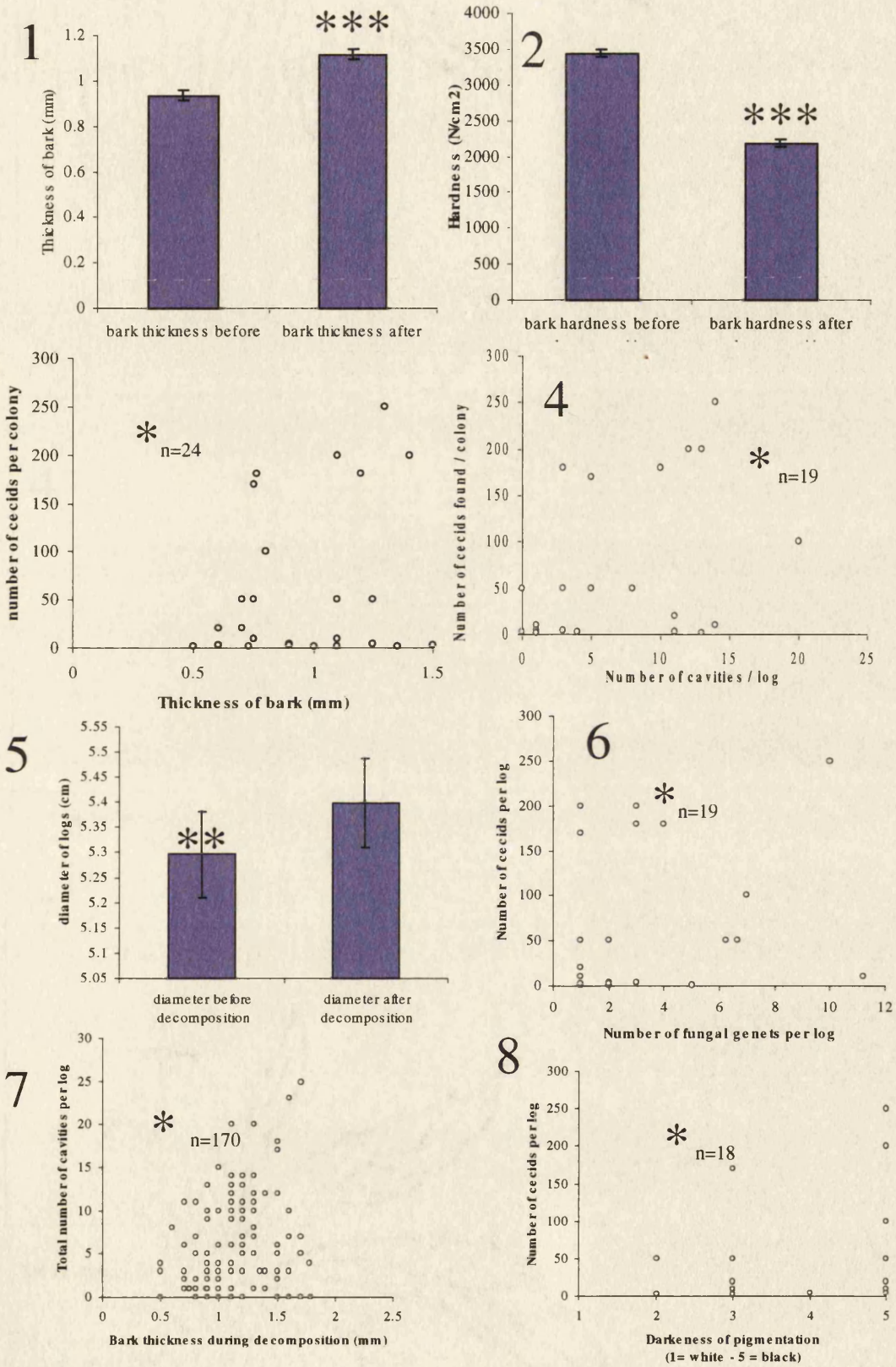


Figure 7.5 Histograms in blue (1,2 & 5) show effects of the decomposition process on measurable features of experimental logs (n=170, *** = P<0.0001, ** = P<0.005, * = P<0.05). Correlations show relationships between measurable factors (P <0.05). All histograms presented in this figure were t-tested to showing significant difference.

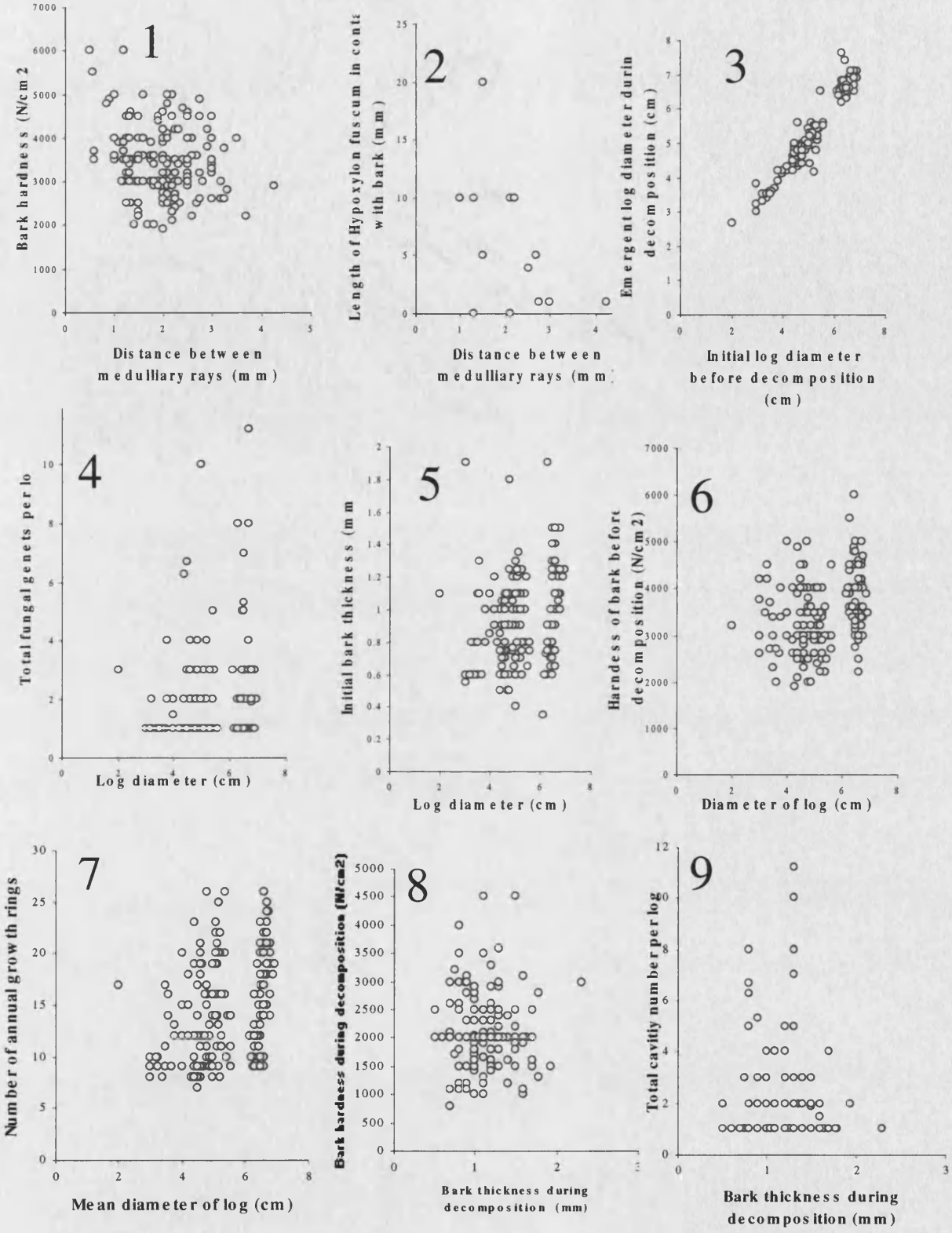


Figure 7.6 Correlation significance between measurable factors in wood slice experiment; (1) n=170, P=0.005, (2) n=13, P=0.05, (3) n=170, P<0.0005, (4) n=58,P<0.05, (5) n=170, P<0.005, (6) n=170, P<0.005, (7) n=170, P<0.001, (8) n=170, P<0.5, (9) n=54, P<1.

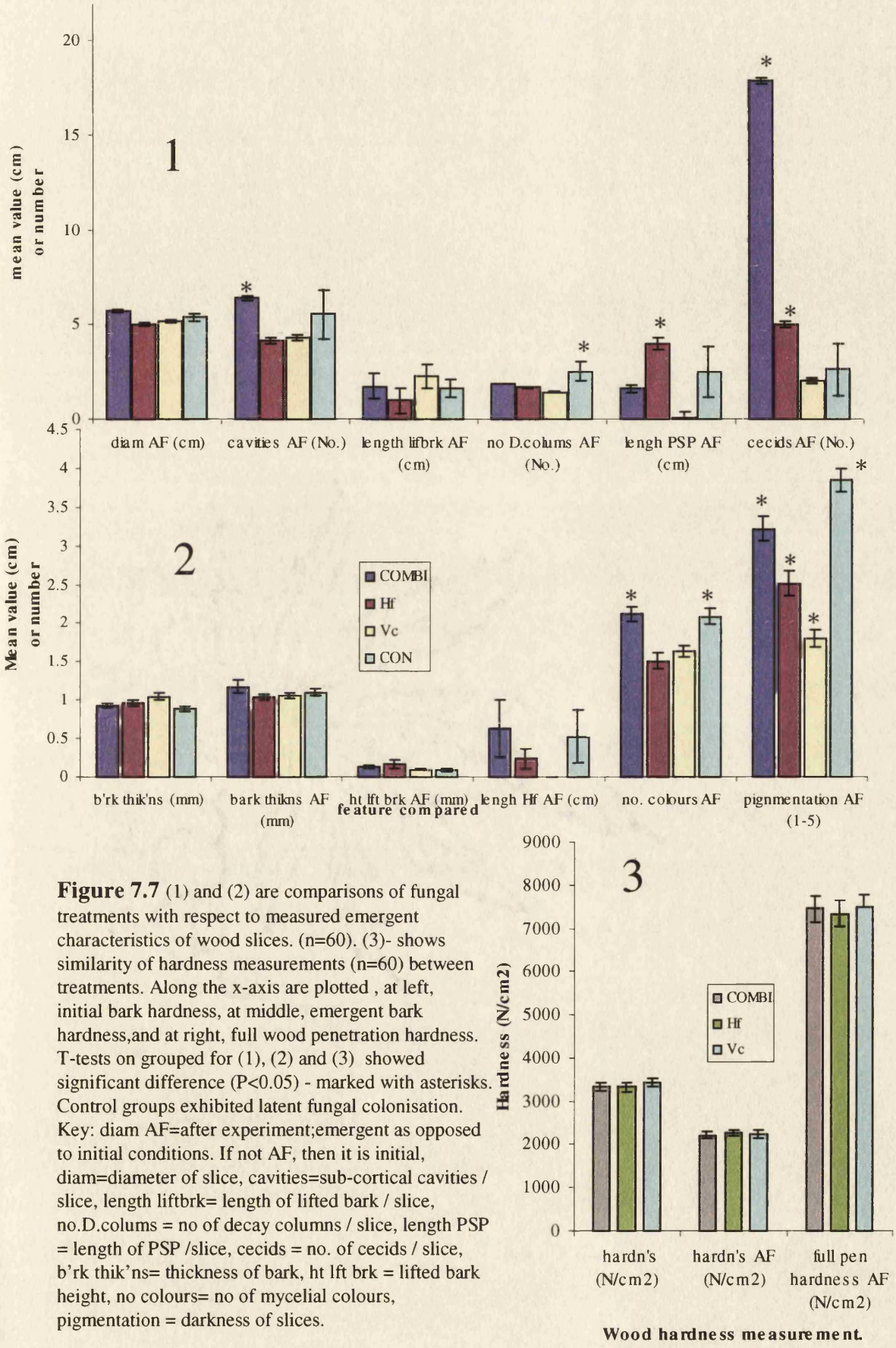


Figure 7.7 (1) and (2) are comparisons of fungal treatments with respect to measured emergent characteristics of wood slices. (n=60). (3)- shows similarity of hardness measurements (n=60) between treatments. Along the x-axis are plotted , at left, initial bark hardness, at middle, emergent bark hardness,and at right, full wood penetration hardness. T-tests on grouped for (1), (2) and (3) showed significant difference ($P<0.05$) - marked with asterisks. Control groups exhibited latent fungal colonisation. Key: diam AF=after experiment;emergent as opposed to initial conditions. If not AF, then it is initial, diam=diameter of slice, cavities=sub-cortical cavities / slice, length liftbrk= length of lifted bark / slice, no.D.colums = no of decay columns / slice, length PSP = length of PSP /slice, cecids = no. of cecids / slice, b'rk thik'ns= thickness of bark, ht lft brk = lifted bark height, no colours= no of mycelial colours, pigmentation = darkness of slices.

Figure 7.5 shows significant t-test differences between initial and emergent data sets as well as significant correlations involving emergent cecid populations within bark layers of wood slices and other factors. Bark is seen to have become thicker and softer and the diameter of wood slices is increased. Increasing cecid numbers are strongly related to the thickness of bark, the number of cavities in bark, the number of decay columns (genets) within wood slices and the amount of darkening of wood layers. Also a strong relationship is shown between the cavity number and the emergent thickness of bark.

Figure 7.6 shows seven significant correlation trends ($P<0.05$) and other near-significant scatter plots. Figure 7.7 shows amalgamated data t-test analyses showing significance where asterisks are present. Raw data tables of correlations between all initial conditions are shown in Appendix 11 tables 7.1 to 7.5. All possible separately performed correlations between emergent conditions are shown in Appendix 11 tables 7.6 to 7.10. Finally, and most importantly, are shown all possible correlations between initial and emergent conditions are shown in Appendix 11 tables 7.11 to 7.15. These tables show all possible correlations between all initial and emergent variables in terms of strength of slope and probability of the scatter plot being due to chance alone. **Numbers in bold are comparisons which showed either strength of slope (>0.10) or significant relationship (P<0.05). Only these were given an asterisk *.**

Tables in Appendix 11 start with initial conditions (and correlations between), then emergent conditions (and correlations between), then finally initial and emergent (and correlations between). One set of such tables is presented for each of the four fungal treatments plus a set for amalgamated results of all treatments together: VC = *V. commedens*, HF = *H. fuscum*, Combi = HF + VC, CON = latently colonised logs, finally ALL = amalgamated data from the four treatments. It was only in light of all of the separately performed correlations that the most significant and important relations could be brought to light.

7.4.2 HPLC results

Figures 7.8 and 7.9 show high-pressure liquid chromatography (HPLC) results. It can be seen that the triple peak signature for interactive cultures and PSP zones (see chapters 5 and 6), which appears in solvent gradient system 2 profiles between 12.5 and 13.5 minutes (lower profile), is found in wood at PSP zones between *H. fuscum* and *V. commedens*, but not found in wood within a *H. fuscum* decay column (i.e. away from any interface). *Corylus avellana* (hazel) wood generally exhibits far more non-polar compounds which produce large peaks in *H. fuscum* decay columns at 19, 21, 25 and 22.5 minutes (top profile) than are produced by fungi growing in malt agar. Many of these non-polar peaks are absent from or drastically reduced in hazel wood profiles from PSP zones (middle profile).

Figure 7.9 shows HPLC profiles to compare extracts from hazel sapwood, hazel bark, *H. fuscum* colonised hazel and extracts of liquorice root bark, liquorice root pith and liquorice resin (commercial). Metabolite peaks at 13 minutes present in hazel sapwood are lost in bark profiles. Liquorice resin and root pith show candidate peaks between 12.5 and 13.5 minutes, which coincides with the triple peak signature of interactive and PSP zones between *H. fuscum* and *V. commedens* (from solvent gradient system 2). Structurally similar compounds between liquorice and hazel are circled. There is more structural similarity between liquorice and hazel than liquorice and pure *H. fuscum* metabolites shown in chapter 4.

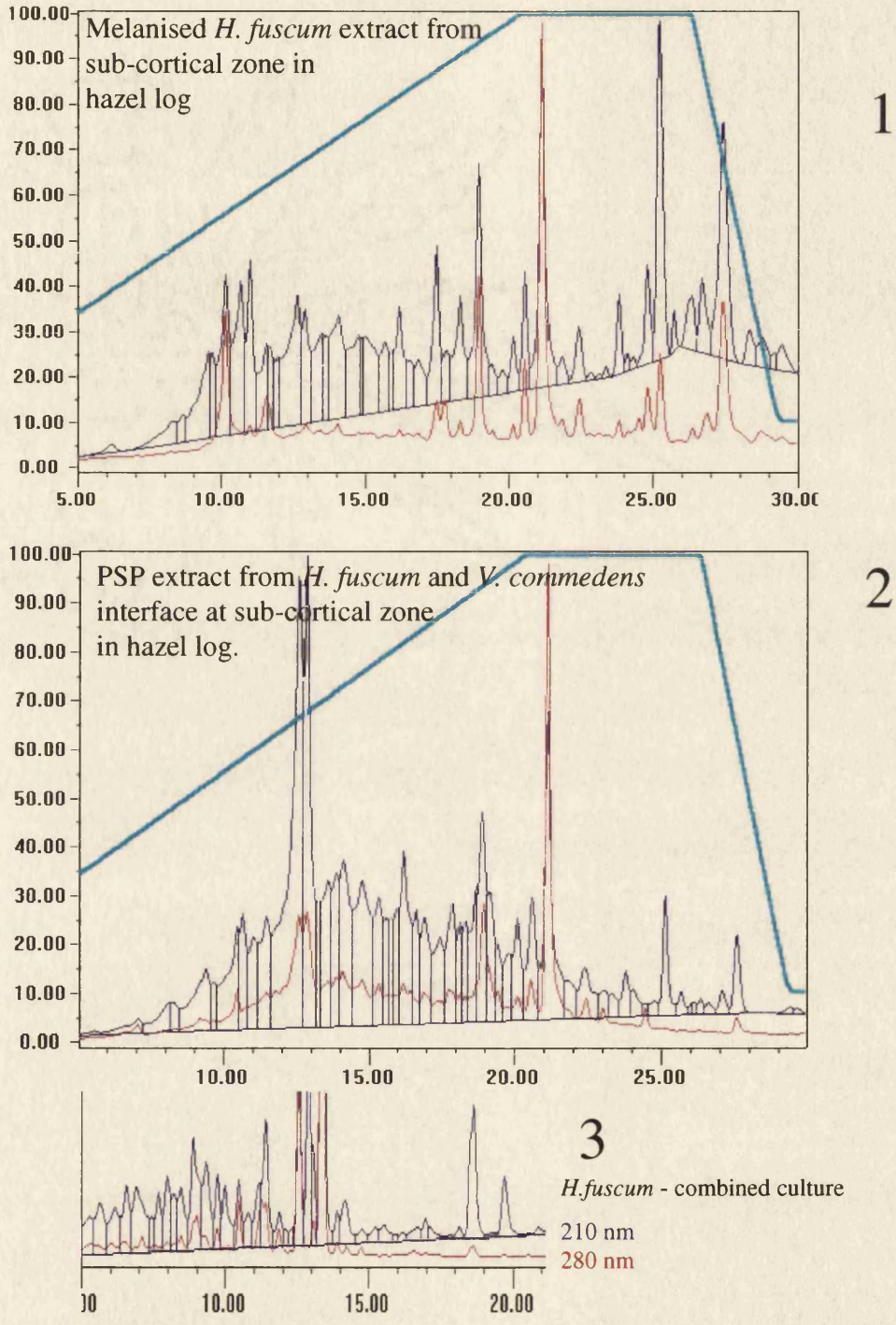


Figure 7.8 HPLC metabolite runs of (1) *H. fuscum* pigmentation from wood of *Corylus avellana*. (2) PSP zone from in-wood interaction of *H. fuscum* and *V. commedens*. (3) Interactive *H. fuscum* from petri-dish culture (for comparison).

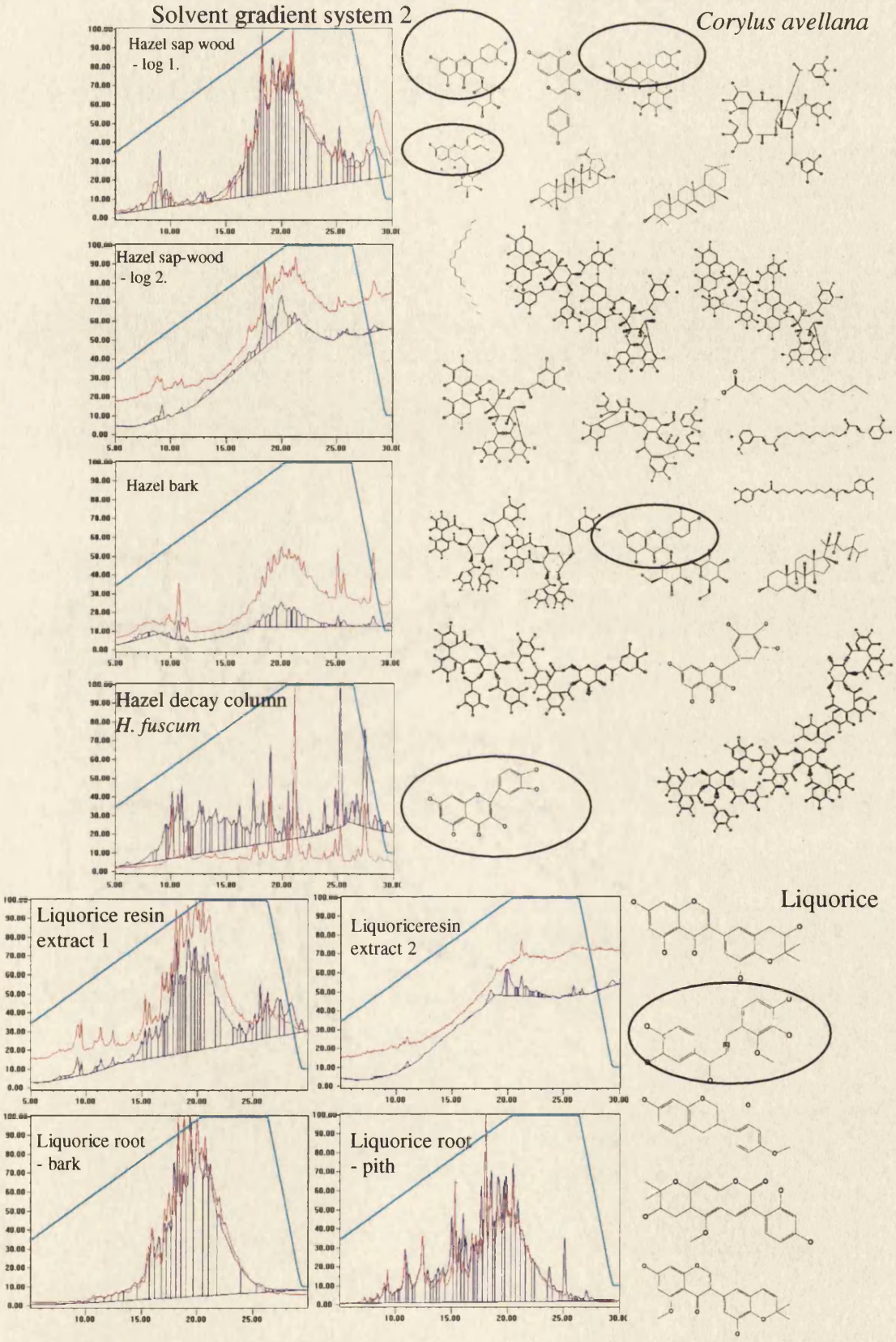


Figure 7.9 HPLC metabolite profiles comparing hazel extracts from bark, sap-wood and *H. fuscum* decay columns, and liquorice extracts from plant root and resin. Chemical structures of known metabolites from both hazel (*Corylus* sp.) and liquorice are shown respectively at right.

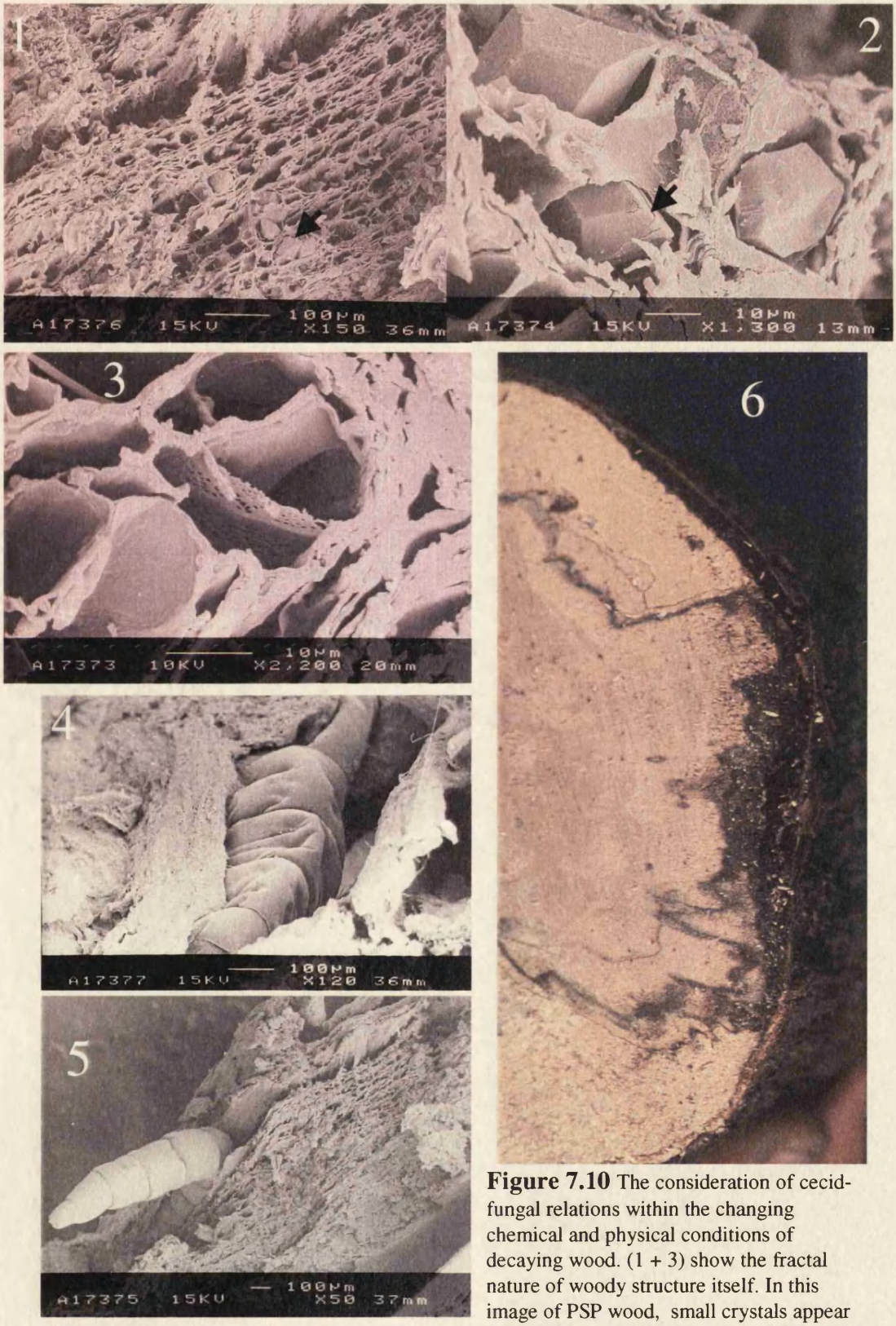


Figure 7.10 The consideration of cecid-fungal relations within the changing chemical and physical conditions of decaying wood. (1 + 3) show the fractal nature of woody structure itself. In this image of PSP wood, small crystals appear at the ends of woody vessels (arrowed). A close up in (2) shows these crystals to be polygonal in shape and covered in a thin crust-like coating (arrowed). (4) shows a young *B. fraxinicola* 1st instar larva negotiating a fine crevice between the bark and sap-wood surfaces. (5) shows a cecid inside a tiny cavity between two of the 3 layers of hazel bark. (6) shows PSP thickening in a log's cross section at the interface with sub-cortical zone and air.

Figure 7.10 shows scanning electron microscopy of hazel wood with PSP zone and cecids within emergent cavities thereof. The structure of woody conduits can be seen to be fractal and the surface area thus dependent on the scale with which the architecture is measured. Fissures in which first-instar *B. fraxinicola* larvae have managed to fit are seen. Crystals of a pigmented chemical are seen in PSP zones. The crystals protrude from resin-filled xylem conduits within PSP interfaces.

7.5 Discussion

Results show that different fungal communities and the presence or absence of cecid larvae significantly influenced the substantial changes that took place in the physical-chemical properties of wood during decomposition.

7.5.1 Wood-decay within an interactive tripartite community

Different fungal treatments of wood slices significantly influenced the emergence of fungal communities and the emergent cavitation within wood slices. In that sense, treatments were successful in influencing wood slices with increased potential to exhibit decay columns of particular treatment fungi. However, these then became part of a wider fungal community including many latently colonising fungi. The result demonstrates that despite two months of treatment between agar colonies of fungi, the latent fungal colonies which were already seeded within living hazel poles were able not only to maintain a footing but to emerge as dominant over experimental treatments with exogenous fungi. This may be explained by the fact that, to a microbe, a piece of wood represents an enormous surface area since hyphae can grow down and in-between hollow xylem and phloem vessels. As such, the latent inoculum held within a small piece of wood might be much larger than the smaller flat agar colonies to which the pieces of wood were subjected for fungal treatment. This also suggests that the degree of latent colonisation of living hazel wood, and perhaps wood in general, could be very high. Despite the fact that the experimental treatments did not oust the latent fungi within any of the live hazel sections, the fact that treatment fungi were indeed present in emergent fungal communities at all shows that the fungal treatments did, at least, alter the composition of the wood slice fungal communities. Therefore, overall the treatments were valid.

Cavity spaces emerged in wood colonised by more darkly pigmented fungi and by combination treated fungal colonisation, with the highest amount of PSP. The more extensive the cavitation, in terms of length of cavities, height of cavities and number of cavities, and the greater the length of PSP next to the bark and overall pigmentation, the more cecids were found on the wood slices. However the decline in both bark and wood hardness was not significantly influenced by treatments. This suggests that cavitation is strongly linked to the decline in hardness, but is an emergent property, which takes place using the softer wood as initial conditions. In other words one cannot predict cavitation from declining wood hardness alone. This suggests that it is the interactivity of the biological factors involved in declining wood hardness that seems to determine when cavitation occurs. If cecids are present during this process, it seems possible that they influence the chemical interactivity of fungi and thus the composition of pigmentation and PSP within wood, also influencing cavity formation through bark loosening. The influence of cecids on chemical properties of fungal interactivity could thus be described as direct, whilst the influence of cecids on physical properties such as cavity formation, which emerge as a consequence of bark detachment, could be described as indirect (Hatcher & Ayres 1997).

The existence of so many significant correlations with *H. fuscum* treated logs, but fewer with any of the other treated logs, suggests that wood slices with interactive zones, also dominated by *H. fuscum* were those on which the potential for significant physical change was greatest. This suggests that the ascomycete *H. fuscum*, through its chemical versatility (chapter 1), had a greater physical effect on the hazel wood. This may help explain the field observations with such high association of cecids to this ruderally-selected fungal species. The strong association of invertebrates with ascomycetes has been noted before by Blackwell (1996).

7.5.2. *Corylus*-PSP scanning electron microscopy

The observation of first-instar cecid larvae trapped very tightly between the sides of cavities in which they have expanded (such that indentations in cavity shape coincide with indentations of the larval cuticle) suggests that there may be physical as well as chemical perception of stress which can trigger paedogenic reproduction. This would make sense for organisms that have evolved to live in such a matrix of cavity spaces and sizes under lifting bark of fungal colonised wood. The first-instar larvae, which find themselves in a roomy cavity, may soon outgrow the dimensions of the cavity in which they found themselves. Cecids, which are effectively "trapped" by their own growth, are then faced with options of paedogenic reproduction or movement to find a new cavity with mycelium. Perhaps forcing paedogenic reproduction could be achieved by larval growth-induced entrapment, which could provide a spatial mechanism to explain asexual semelparous paedogenesis (Keeling & Rand 1995, Ranta, Tesar, Alaja, Kaitala 2000). The mechanism of such physical paedogenic forcing may be chemical (i.e. oxidatively-induced) since if the larvae are trapped they may well run out of available mycelium in which to feed, which could cause larval stress levels to increase internally. Even if the larvae manage to free themselves, they may be too large to easily find other available cavities in which there is still some room for them to fit. On the other hand the benefits of paedogenic reproduction maximise chances of at least one small daughter larva finding an available cavity somewhere in the matrix. This may be achieved through thigmotropic response to touch (Fraenkel 1961, Wyatt 2000).

This scenario opens the intriguing possibility that cecid larvae may expand in cavities much like balloons and actually apply pressure to the walls of the cavity. Incremental increase of cecid-induced cavity forcing at a population level, and over the time course of decomposition processes, may act overall as one of the factors that contribute to cavity formation in the bark matrix. Similarly, but at a much finer scale, the polygonal crystals of unknown chemical composition which are exclusive to PSP zones may also expand as they grow within and between xylem conduits and thus provide a force which fragments previously latticed and toughly bonded components of cell walls and inter- and intra-vessel bonding materials. Polygonal crystals, observed in wood but not in agar cultures, may provide one of the chemically induced links to a physical set of changes within decomposing wood. The crystals, which could possibly be oxalate (Dutton & Evans 1996, Whatley, Sayer & Gadd 1998), contribute further evidence of synergistic chemicals leading to synergetic force (self-organised physical events) (Haken 1980).

Oxalate is a good potential candidate for these crystals since it has been discovered in a wide variety of antioxidant metabolic pathways involving the conversion of oxalic acid to an oxalate such as calcium oxalate. Oxalate metabolism is known to be very important to fungi in their ecology and roles as

decomposers. It has been suggested that oxalic acid is produced by all classes of fungi (Dutton & Evans 1996). Moreover, it has been shown that oxalate crystals occur inside xylem vessels and also often appear covered in a thin material, which has been shown to consist of fungal wall residues (Dutton & Evans 1996), as were the crystals I found shown in figure 7.10 (2). It has been shown that crystals of calcium oxalate weaken cell walls and act in concert with free radical producing enzymes, such as polygalacturonase, to effect degradation. It is thought that oxalate dissolves nutrients especially Fe and Al. Oxalate also provides a reservoir of calcium in ecosystems (Dutton & Evans 1996). Oxalate possesses a metabolic ability to sequester free radicals into a crystalline form from oxalic acid precursor. Also, important to wood-decay fungi, oxalate is thought to play an important role in lignin decomposition releasing free radicals, oxygen, H_2O_2 and CO_2 in the process. Moreover, oxalate is known to be involved as a co-factor in the regulation of two of the key enzymes investigated in this thesis, peroxidase and catalase. The production of crystals of synergistically produced novel fungal metabolites has been noted before in the emergence of + torreol from somatically incompatible reactions of isolates of the basidiomycete *Stereum hirsutum* (Ainsworth *et al.* 1990).

7.5.3. Metabolites of *Hypoxylon*-PSP-*Vuilleminia*-*Corylus* system

The existence of a specific PSP chemical environment has been shown. This, together with the other chemicals that are found only in wood, may contribute to the formation of darkly coloured PSP crystals. The propensity for PSP chemicals to loosen bark layers and to loosen the connectivity of medullary ray to inter-medullary ray wood fibres, explains both the increased potential for cavitation through bark detachment at PSP zones, and also why cecids may find it advantageous to inhabit these regions. The maximum physical potential for larger size classes of cavity and cecid larval sizes to emerge under bark (Taylor 1999 b) are found in these regions. **The emergence of a sub-cortical cavity matrix architecture, through the realisation of combined physical and chemical potentials and also influenced by the organisms which inhabit them, is the topic of the synthesis presented in chapter 8.**

CHAPTER 8: DISCUSSION INCLUDING A GENERAL SCHEMA FOR UNDERSTANDING THE RELATIONSHIP BETWEEN INSECT AND FUNGAL ACTIVITY WITH RESPECT TO BARK DETACHMENT AND DECOMPOSITION DYNAMICS

8.1. Synopsis

The case is made that dead-wood-inhabiting insect larvae act as agents of perturbation in dynamic systems of interactive co-existence between members of the fungal wood-decay community. The emergent fungal community patterns affect development of insects and their populations as described in a cycle of developmental feedback. Evidence of such reciprocity of interaction stems from the case study of paedogenic cecid larva *Brittenia fraxinicola* and the particular relationship this insect has between interactive states of the ascomycete *Hypoxylon fuscum* with members of hazel's (*Corylus avellana*) basidiomycete fungal community, in particular the fungus *Vuilleminia commedens*. Subsequent emergence of distribution patterns observed between insects and fungi of the sub-cortical zone is explored through a synthesis of available evidence gleaned from the previous 7 chapters. The developmental processes producing emergent distribution patterns centre on the role of reciprocal feedbacks between insects and fungi and fungi and insects across their interactive boundaries which are sensitive to and directly changed by levels of **chemical oxidative stress** - providing evidence of a **chemical potential** which exists in this system. Boundary surface sensitivity to oxidative stress leads to changes in interface chemistry that includes **synergistic production of completely new compounds** within pseudosclerotial plate (PSP) zones. Within wood and, to a lesser extent agar, these chemical synergies result in a **progressive physical emergence**. Thus, a **physical potential** is provided indirectly as an emergent property from composite chemical synergy that seeds it. **Wood cavitation**, a physical property that is influenced but not controlled by cecids, is provided by bark expansion, loosening and finally detachment and lift within PSP and interactive *H. fuscum* domains. Cecids act as chemical perturbers and channel the physical processes in this dynamic system to their advantage. *B. fraxinicola* larvae mediate as vectors of a reciprocal exchange of chemical and enzymatic redox agents between interactive states of basidiomycete and ascomycete interactivity. In so doing, the larvae influence both chemical and physical potentials to their advantage - reducing oxidative stress and increasing the production of PSP zone. Cecids have evidently adapted their foraging behaviour, reproduction, dispersal and genetics specifically to influence the emergent architecture of the fungal / wood interaction.

8.2 Introduction

Previous chapters have given an account of cecid-fungal-wood interactions in a local woodland, and then worked through seven scales of observation and measurement. In this chapter I synthesise incrementally accumulated scales of understanding that have been obtained. I also attempt to widen the scope of this project's results to link with other classic and more recent empirical and conceptual work (Fisher 1937, 1938, 1940, 1941, Fisher & Parkin 1930, Hickin 1975, Cooke & Rayner 1984, Anderson, Rayner & Walton 1984, Swift & Boddy 1984, Dowding 1984, Rayner 1997, Hatcher & Ayres 1997). This is so that a case can be made in general for the accommodation of insect larvae in dead wood fungal communities. This case is made

using cecids, in particular *B. fraxinicola* larvae and their relations with interactive states of the ascomycete *H. fuscum* and basidiomycete members of the hazel (*Corylus avellana*) decay community, in particular *V. commedens*. The relevance of developmental feedback in enhancing the potential for emergence of synergistic chemical and cavity patterns will be discussed in terms of implications for understanding processes such as symbiosis, biodiversity co-existence, succession, co-evolution, evolutionary adaptive processes and conservation. Regarding the case study of cecid larvae of *B. fraxinicola* and their relations with fungal interfaces (chapter 3), it is now possible to piece together the chemical feedback processes (chapters 4, 5 and 6) as influenced by *B. fraxinicola* larvae, to form patterns in decomposing wood. Moreover, to examine key findings in light of the role of chemical synergy (Salvador 2000, Sondheimer & Simeone 1970, Haken 1980, Ainsworth *et al.* 1990) in the emergence of a physically structured PSP zone (chapter 7). The resultant bark-lift induced cavitation fits into a general schema of spatio-temporal events in the sub-cortical zone of hazel bark. **The overall philosophical implication of this work emphasises the importance of the environment in the process of feedback and reciprocal interactivity between living entities. Reciprocally interactive states change boundary conditions at the reactive interfaces of organisms. Changes in boundary conditions effect chemical synergies and emergent physical potentials, channelling developmental form at many scales.**

8.3 General discussion

8.3.1 The synthesis- towards a general schema

Field and laboratory observations presented in chapters 3 to 6 provide clear evidence of a complex interplay between the distribution and activity of insects and fungi within the primary decay of hazel poles. In effect, fungi provide a dynamic context for development of insects that is in turn effected by the activity of the insects themselves. Understanding such complex interplays and their repercussions over ranges of scales is vital to development of methodologies that characterise and compare natural communities in meaningful ways. The interplay between insects and fungi could be used as a case study of the effects of perturbation-triggered feedback on the biology of complex interactive dynamic as opposed to simple isolated less interactive states of chemical and physical potentials (Herbert 1983, Hanski 1999, Prigogene 1980).

As discussed in chapters 2 and 3, approaches to scale are important considerations within ecological science. The approach which has been refined during the course of this project is perhaps best summarised in the following statement: "**Whether the question of study be categorisation, distribution or basis of symbiotic interplay, questions in complex ecological systems are perhaps investigated most meaningfully by observing, experimenting and interpreting from a range of scales and perspectives.**"

Through this approach a simple standard protocol enabled fungal biodiversity to be effectively compared in the field in Canada. This technique was adapted in the UK to investigate patterns of fungal biodiversity and provided a key role through which to understand the processes involved in creating patterns of fungal community interactions with insects under the bark layers of decaying wood.

Regarding the case study of *Brittenia fraxinicola* cecid larvae within the fungal community of decaying *Corylus avellana*, a sequence of events has emerged from empirical data presented in chapters 3 to 7 which sheds light on the chemical potential for emergence of physical conditions within three layers of hazel bark.

Figure 8.1 represents a view of the fungus wood cecid system in cross-section. The thin black lines represent wood colonised by *Hypoxylon fuscum* and red shapes show *B. fraxinicola* first and second-instars and positions in cavities within bark or on the sapwood surface. Thick black lines represent pseudosclerotial plate (PSP) zones of cecid-enhanced melanisation (chapter 5). Green indicates dynamic changes taking place - firstly **(1)** the initial conditions and variable structure of the living wood before development of latent fungi, age and expansion related corrugation sizes and distances between medullary rays and bark thickness (chapter 7). As soon as wood dies and latent fungi become active, a chemical potential of interactive responses (chapter 6) between fungi enhances the physical probability of bark layer loosening and expansion (a physical potential). These softer bark layers may be ruptured by fungal fruiting bodies **(2)** where bark may begin to peel and lift **(3)** creating new cavities (extended physical potential) for smaller explorative larvae (as shown in chapter 3). First-instar larvae colonise vacant cavities within which interactive mycelia form PSP zones that provide refuge from oxidative stress present in interactive mycelia. Here the larvae enhance a reciprocal metabolic exchange (see chapter 6) across the developing PSP zone and thereby increase the chemical potential for synergistic metabolic products (see chapter 6) between interactive mycelia. This chemical synergy leads to a decrease in reactive oxygen intermediates (ROI), a thickening of PSP zone and an increased physical potential for cavitation within the developing bark-PSP complex (see chapter 7). The smallest and newest cavities are inhabited by the smallest larval offspring (see chapter 3). As interactive fungal and cecid colonisation permeates the bark matrix, gaps emerge between softened and widened layers **(4)** in conjunction with cecid-enhanced melanisation **(5)** so presenting to each successive generation of expansive larval shapes (chapter 7) a variety of spaces into which to move by thigmotropism (chapters 3 and 7). This strengthens observations by Springer and Kahle (Springer 1915, Kahle 1908). Results from field and lab work combined also point to a trade-off that is associated with the need for larvae to minimise time spent in contact with their food source. Nutritive mycelia are rewarding food sources, but because they are rich in ROI, they impose a cost with increased larval contact. PSP zones, on the other hand, represent hydrophobin-rich domains that are free from hyphal lignin catabolism from which free radicals are released. Cecids may feed in one or both sets of interactive mycelia, but their foraging loops soon bring them back to the PSP zones (figure 5.4) where they find a reduced amount of reactive oxygen species (ROI) but also a lack of food. Once at the PSP zone, the cecids again face a trade-off between a less hostile environment, which possesses little by way of nutrition due to its lack of active hyphae.

B. fraxinicola strategies to reduce the harmful effects of free radicals and other secondary fungal chemicals may require metabolic energy, and take energy away from potential larval growth, in a similar way to the effect of resistance to insecticides and natural plant defence compounds such as terpenes, tannins and alkaloids (Waller & Dermer 1981, Haslam 1981, Georghio & Saito 1983, Kleepzig, Kruger, Smalley & Raffa 1995). It is thought that insecticide resistance mechanisms have energetic costs that, in the absence of selection pressure, maintain a low frequency of the resistance-conferring mutations in comparison with wild-type genotypes. Thus it may be energetically adaptive for *B. fraxinicola* to return to PSP areas of low oxidative stress as often as possible after feeding, and for as long as possible between feeds, which would reduce the penalty of oxidative stress. This idea is further strengthened by the observation of mothers, which feed and forage less actively than first-instars, spending proportionally more time at PSP zones, prior to giving birth there. Moreover, PSP zones would be the least metabolically hostile places for long term dormancy in summer months under bark as resting mothers (Wyatt 2000).

The work on cecid paedogenesis by Hunt (1996) found no evidence of larval parasitism on mother tissues prior to paedogenic birth. This suggests that mother larvae do not, as was previously thought, passively carry their spontaneously developing larvae. Instead there is an alternative school of thought that developmental feedback is involved between the larval chemical and nutritive context and the mother's initiation of follicle development (Went 1973, 1975, 1977, 1979, Went & Camenzind 1977). It is possible that the chemical and nutritive environment, together with the size mothers have reached by the time oxidative stress accumulates, determines how many follicles become activated and how soon after birth this activation begins (Matuszswski & Zagrodzinska 1994). Thus I suggest that nutrition and oxidative stress act in concert as part of a causal event network. The hypothesis emerging from this is "That the size a paedogenic cecid larva has reached by the time she reaches motherhood is proportional to the number of follicles which are activated." This would result in the observed trend of interactive cultures with larger mean cecid sizes giving rise to larger population sizes and pure cultures with smaller mean cecid sizes giving rise to smaller populations.

Such adaptation to fungal chemical ecology (Sondheimer & Simeone 1970) may relate cecid genetic polyploidy to mitotic follicle induction. Such a process may enable cecids to select which of the many sets of chromosome copies remain suppressed ("eliminated") and which become used by larval cells (White 1946, Brodsky & Urvaeva 1985, Fux 1974, Hardy 1964, Itami, Craig & Horner 1998). This type of chromosomal mitotic elimination may especially function at times of stress to increase the genetic diversity of offspring without the costs of a full sexual cycle, in much the same way as it does in some polyploidous plants (McClintock 1983, Hodin 2000, Hodin & Riddiford 2000b).

Relating the above to an evolutionary explanation, such a mechanism of heterogeneous environmental feedback could perhaps have been selected to produce observed patterns through a process of larval sensitivity to the level of oxidative stress whilst feeding (Godfrey, Muller & Kraaijeveld 1999). Larvae are covered in sensoria (chapter 3). In this scenario, more highly oxidatively stressed larvae can only find a way out of the metabolic environment by locating a PSP zone or by climbing out of agar onto the edges of the Petri dish (a common observation in pure cultures).

In oxidatively stressed environments perhaps mothers have adapted by giving rise to as much diversity as possible through polyploidous mitotic chromosome elimination reassortments (the stress-induced diversity-inducing trick available to some organisms with high polyploidy). This results in fewer larvae being born but possessing increased genetic diversity via a genetic-metabolic interface which is likely to be similar to that found in other organisms with high polyploidy (McClintock 1983, Kaiser & Went 1987, Kahle 1908, Hodin 2000, Hodin & Riddiford 2000 a, Soltis & Soltis 1999). The development of this spatially influenced semelparous reproductive system seems to be environmentally determined (Ranta *et al.* 2000). Each larva has the potential to disperse and found a new colony (Soltis & Soltis 1999). The increased genetic diversity of dispersing cecids increases chances of successful dispersal since the increased diversity of larvae increases the chances of survival in any newly located niche. The founder effect, acting on this diversity, allows a greater amount of adaptation than would be the case otherwise (Hardy 1964).

The converse strategy is that, in more favourable sites where oxidative stress is less damaging, paedogenic larvae grow bigger before initiating follicle development. These follicles, possibly as a consequence of less stress-induced transposition events (Mc Clintock 1983) are perhaps more genetically faithful to the mother's

particular polyploidal chromosome selection (a feature of very large polyploidy is the use of only a particular selection of the chromosomal copies available in each cell). Thus the developing embryos are more genetically uniform. In the synthesis presented here, I speculate that there is a process by which the size of the mother allows a greater number of cecids to be accommodated within her (observed by Camenzind, 1982). The result is larger mothers giving rise to more numerous and genetically uniform larval offspring in environments with less oxidative stress (Springer 1915, Wyatt 1960).

Figure 8.2 outlines how the above findings relate to the overall context of insect-fungal interactions during wood decomposition and soil formation. To view figure 8.2 please consider the range of scales through which ecological successions and decompositions occur. Add to this the expansive nature of niches over time (Bowers 1994) in terms of their scales (Duarte, Boldrini, dos Reis 1998), the expansion of living wood (Dowding 1984), intermedullary ray distances, sapwood corrugations and bark layers. A synthesis emerges in which the range in scale of surface area available to organisms varies from living wood (top left), dead wood (top middle), through decomposition, compartmentalisation, cavitation, fragmentation into soil by successive fungi and insects of different scales of succession from ruderal towards K-selected (green), and so back into roots and into living trees (top right). Under each of the vertical-dashed green lines are probability volumes - ranges of scales of, for example cavity-size in blue indicating that through co-existent cavity-size allocation, fungal and insect successions may be linked (Smith 1960), or thickness of bark (black), or general ecological surface area in red, which one might expect to encounter from left to right across the top schematic, and over time. The long black triangle two-thirds of the way up figure 8.2 highlights the vital contribution accumulated fungal PSP makes to humus content and soil formation, through the production of melanised surface pigments which are influenced by insect larvae and other invertebrates which live inside fungal interfaces. Fungi here appear to be the fundamental bridge between successive peaks in ecological scale of surfaces of living trees (Rayner 1993 b, 1997, 1999). They enable continuity between passing away and entering into being and help create the very cavities through which successive scales of insects and other animals may be found. In this way fungal organisms are inclusive in the environment that affects so much of the development of forest ecosystem structure, which affects the potential to accommodate insect and other animal biodiversity (Davies *et al.* 1998).

A succession of decompositional fungi and associated cavity-sizes has been suggested by cavity-size measurements in chapter 3 (Frankland 1992). Given the propensity to find cecid larvae within appropriately sized cavities which were themselves the emergent outcome from a relationship between two particularly ruderal fungal species, it seems likely that the successions of fungi and insects tend to become synchronised. Thus fungi create physical potential for emergent cavity-size classes that fit the insect species living in them. Moreover, successive waves of fungal decomposition interact, causing cavity-size expansion, and so the place where cecid larvae are most likely to be found (Eidmann 1964, Eichhom 1991) moves spatio-temporally along a rotting branch to keep up with the migration of pioneer colonising, more ruderal wood-decay fungal species.

If insects and fungi can be associated through their chemical and physical reciprocal effects on each other, there is good reason to suspect that this reciprocity represents a mechanism for the evolution of specific co-existence relations amongst interactive partners co-existing in decomposing wood (Tokeshi 1999, Pirozynski 1988). As decomposition progresses, so the successive waves of both fungal community and insect

perturbers become more K-selected and cavities widen (Clift & Terras 1995, Davies *et al.* 1998). The ability of fungi to assimilate both water and nutrients at one end of a mycelium and trans-locate these to another area of the network is widely known, most famously in dry-rot *Serpula lacrymans*. However, the importance of water ecology may be an ecological dimension that has not been fully recognised by developmental biologists in terms of the diverse strategies which species employ to conserve, move, assimilate and defend their internal and external water regimes (Woods & Bernays 2000). Furthermore, the observation of many insect larvae inhabiting such humid sites supports the general hypothesis that insects are attracted to the boundary properties of fungi under bark. The insects may merely be attracted to sites that are the right size for them, are relatively abiotically stable, have food and are also well insulated from stresses caused by too much oxygen, too little water, excessive predation or all of these. It may be that the foraging of insects within these critical fungal boundary zones, which are so important in determining abilities to regulate oxygen and water stress, affects fungal boundary shapes and thickness which reciprocate by affecting fungal oxidative stress levels as felt in protoplasm. Cecids may also introduce bacteria, yeasts and fungal spores (Ingold 1953), as seen in the present work on the scanning electron micrographs of cecid cuticles. If a boundary becomes multiply enfolded, rates of water loss through evaporation may increase, as with a sponge with a high surface area to volume ratio. By the same token, as with the sponge, water assimilation rates may increase during times of temporary water excess, for example during showers in a long hot summer. This may give advantage to those fungal species which can complete more of their life-cycles in times of short periods of wetness interspersed by long periods of dryness - those which are more r-selected. More K-selected species take longer to accomplish substantial events in the life-cycle and consequently require more stable water regimes to last through the duration of a life-cycle change from one insulated form to another. K-selected species will be out competed from microhabitats around which boundaries have been enfolded and thus made more porous. In addition, **inversion of normal ecological r- to K-selected succession may occur when organisms alter their boundary properties in this way to make their contexts more susceptible to influence from surrounding more rapidly oscillating abiotic factor regimes**. Conversely, a thickened boundary (i.e. one which has been re-sealed inside itself) may be the defence K-selected fungi employ against the aforementioned **"r-K succession inversion".** By thickening boundaries despite their enfolding due to insect foraging, the maintenance of an already established K-selected fungus domain within a sealed, stable and more long-termed oscillatory pattern of abiotic factors, may be maintained and so may the succession from r- towards K-selection. All these hypotheses come under the general heading of "water ecology" and are in need of further empirical studies.

Clearly, aside from the importance of water and oxygen and boundaries, one of the conditions to be met within sub-cortical zones is niche dimension - the size of the gaps in sub-cortical complexes must be large enough for inhabitation by the insects in question. Cecids were always found under bark that was loose (as in the case of the PSP pigmentation provided by *H. fuscum*) or peeled back (as in the case of *V. commedens*) enough for a gap to become available. Likewise a 1 cm diameter stag beetle larva *Lucanus cervus* was found in a decay hollow between outer bark and sapwood that was large enough for the insect to be active. This prompts the hypothesis that sub-cortical niche dimension is directly proportional to the dimensions of insects which may eventually inhabit it. Thus a fungus-bark combination which, as with the case of *V. commedens* and *H. fuscum* on *Corylus avellana*, makes available potential conditions for niche space to open up suited to a particular genera of insects.

Figure 8.3 considers the directions of r-K-selection relative to fungal decomposition (as ecological catabolism) or plant community succession, growth and scale expansions (as ecological anabolism) which increase ecosystem surface area (Cobb & Whitham 1998). The succession of ruderal to K-selected species occurs in both processes, but ecological anabolism expands surface area whereas ecological catabolism fragments and compartmentalises it. Here it must be remembered that cecids and associated fungi are decompositional community pioneers. Also that *H. fuscum* is more ruderal than *V. commedens*. Results from chapter 5 using combined fungal cultures indicate that the effect of cecids is to reduce the combative ability of *V. commendens* and perhaps the rate at which this fungus might replace *H. fuscum* in nature. Basidiomycetes are generally seen as more K-selected than the Ascomycotina. Under normal circumstances, the basidiomycete *V. commedens* succeeds over the ascomycete *H. fuscum*. Isolate 2 of *V. commedens* is an example. It produces invasion fronts into the *H. fuscum* mycelial domains. However, the influence of cecid population development across the PSP zones that separate these two fungi seems to reduce the combative abilities of *V. commedens* and thus stabilise the PSP niche (since, if *V. commedens* ousts *H. fuscum* from a piece of woody debris, the PSP zone would be lost). Since the combative ability of *V. commedens* is hindered by cecid perturbations, the overall emergent architecture of woody debris is maintained in a state of higher diversity, higher co-existence and higher interactivity and therefore more PSP zones than would be the case if combative relations between fungi continued. So cecids, and other organisms which exert a similar influence, are perhaps delaying succession and enhancing stability of wood-decay architecture - enabling the time duration of the state of interactivity between two fungal species at the ruderal beginning of hazel decay to be lengthened.

The effects of cecids are thus twofold. Firstly, the action of *H. fuscum* is prolonged, acting in its advantage in so far as relations with *V. commedens* are concerned. Secondly, this implies that in nature, cecids may be capable of prolonging the state of interactivity within a decomposing log, thus maintaining a higher biodiversity, than if *V. commedens* managed to oust the more ruderally selected Ascomycete. This implies that the normal community succession from r towards K-selected is delayed by the activity of PSP-enhancing cecid larvae, which could act to benefit both ruderal Ascomycete and larvae. The implications of this are that the proportions of r- and K-selected species in a woodland ecosystem are in dynamic equilibrium with potential for micro-trends (analogous to back-eddies in a stream which forces some water against the flow) in either direction, whilst the overall macro-trend (analogous to the main current of a stream) leads to a succession towards K-selection. Expanding surface boundary area within an ecosystem at a landscape scale, mirrors the expansion of tree girth, bark thickness and cavity-sizes at smaller scales. This helps to explain resource-partitioning processes. Where two or more different insect species, or founder-effect induced diversity within the same species (Butler, Schoener & Losos 2000), become adapted to cavity-size classes and the chemical synergies (Dillon & Root-Bernstein 1997) of fungi which inhabit them, so they become able to co-exist in the same tree or woody debris without competitive exclusion. The longer the co-existence, the higher the probability of co-evolution towards commensal or mutualistic symbiosis (Cooke & Rayner 1984).

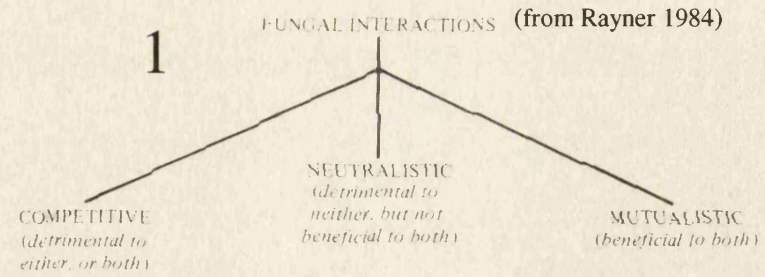
With a fractal range of cavity-sizes available in a piece of woody debris, huge numbers of species of organism, with differing body size, become able to co-exist where, without the existence of cavity boundaries, might otherwise be more likely to compete for space and resources and move towards more combative symbiotic or exclusionist states. Thus there is a conceptual link between co-existence, co-

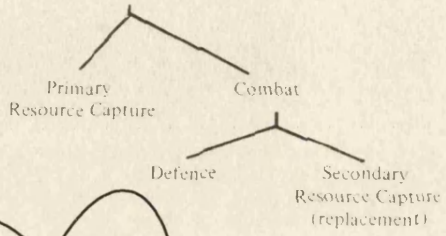
evolution and symbiosis. Organisms that initially are combative may be induced by the environmental architecture to co-inhabit the same space (Mongold, Bennett & Lenski 1996, Wertheim, Sevenster & Eijs 2000). It is thought that co-existence, if long lasting, tends to evolve towards neutralistic, commensal or mutualistic types of symbiosis (Morin 1999, Rayner 1997, Maurer 1999). The key point here is the influence of the environmental morphology or decompositional architecture on the type of symbiotic relations that emerge.

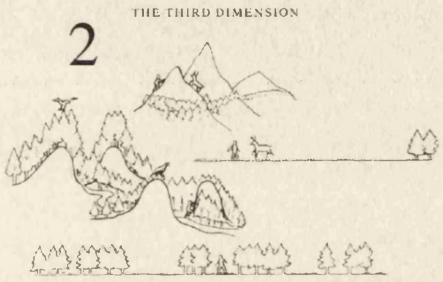
Figure 8.4 – (1) and (2) highlight how relationships between co-existing species may be related to habitat topography. For example, in predator prey interactions, if diversity increases with number of participants or if prey can hide more easily due to geographical or biological landscape features, more stable population dynamics may emerge, increasing the overall co-existence so that populations are less prone to crashes (May 1973, Lotka 1956, Logan & Allen 1992). If species co-existences are long lasting (in relation to life-spans of organisms involved), as with lichens, biofilm formation, and other symbiotic assemblages, the potential for emergence of structure which may itself be colonised is increased. I suggest that the accumulation of structural surface takes place to a greater extent as a result of longer lasting commensal and mutual interactions between organisms, and this surface may be colonised by successions of organisms from ruderal early-pioneer to K-selected, late-stage colonists (Gorbushina 1998, Margulis, Schwartz 1982, Allison 1993).

As the schematic (3) in figure 8.4 illustrates, communities of cobble encrusters (Bogomolva 1998, Tokeshi 1999) from the rocky shore produce the highest diversity and heterogeneity when the extent of interactivity between different stages of community succession is highest. At each extreme of the process, where mono or pure cultures of either ruderal or late-stage K-selected colonisers become dominant, the diversity drops, as does the heterogeneity of the system. **So it appears that the maintenance of a state of interactivity and heterogeneity increases persistence of co-existence** (Bonsall, Jones & Perry 1998), **increases the amount of symbiosis, which, if long lasting, tends away from combative exclusion, and increases the diversity of the system** (Delavega 1996). This process keeps the system in a state of anabolism relative to the participants in the system (i.e. even if their niche changes or decomposes). In the case of cecids, they seem to enlarge the PSP zones by vectoring a reciprocal metabolic exchange from one mycelial domain to the other, thus increasing the extent of interactivity in terms of area, and preventing *V. commedens* invasion fronts from maintaining coherence across PSP zones. The combination of outputs from the maintenance and enhanced degree of interactivity between *H. fuscum* and *V. commedens* seems to lead to an effective increase in niche space available to the cecid population. Such a combination of outputs seems to satisfy multiple niche requirements of *B. fraxinicola* larvae, shown in the Venn diagram in figure 8.4.

Considering each of the Venn diagram factors in an anticlockwise direction from the bottom, the particular architecture that emerges in PSP zones between *H. fuscum* and *V. commedens* under hazel bark seems to satisfy the niche requirements of the range of cavity-size-classes into which different cecid sizes can fit. A network of channels in bark interconnects small, newly forming cavities near the freshest PSP, with larger older cavities near the oldest PSP. In these channels, the availability of, or the presence of water and high humidity could be increased by H_2O_2 breakdown by *H. fuscum* peroxisome catalases releasing $O_{2\,(g)}$ and $H_2O_{(l)}$. In the PSP zone itself, a decrease in predation by parasitic wasps and larger insect larvae could be







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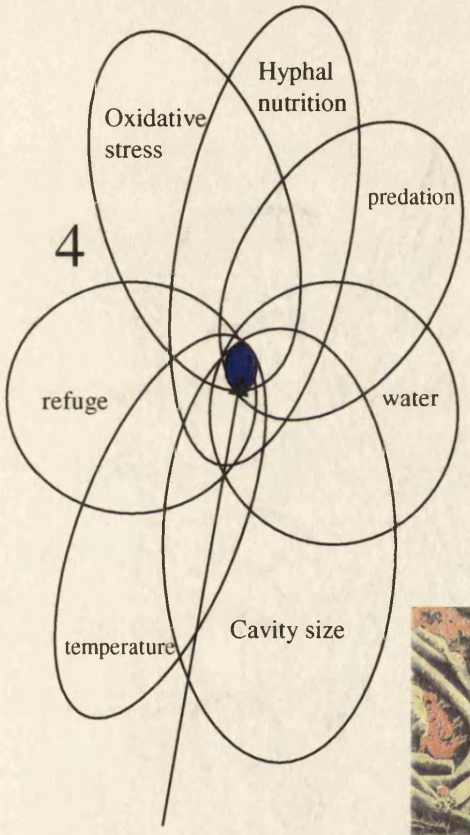

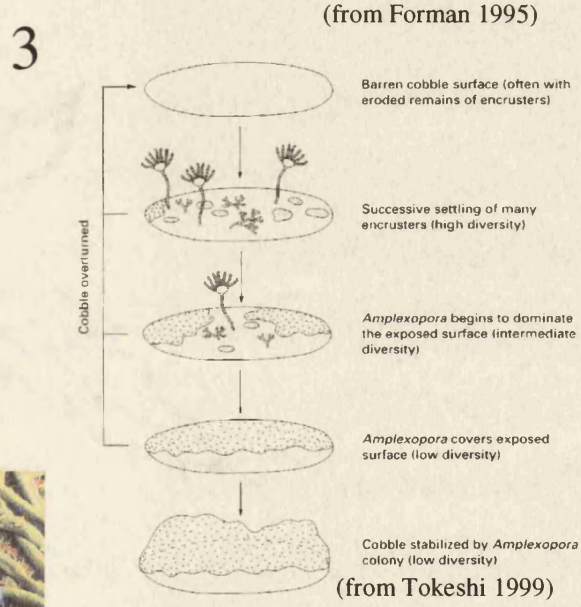

(from Tokeshi 1999)

Understanding cecid abundance distribution and ecology in terms of context.

Figure 8.4 The process of feedback enables symbiosis to occur (1). If long lasting this allows new surface areas to become available for feedback; which drives ecological anabolism (3). Ecological anabolism gives rise to the potential for development of architecture to which species adapt and relate according to their body size (2). Through the process of studying the cecid environment as a case study in understanding an organism's ecology, I consider the potential for finding cecids to be highest where there is maximum overlap between the factors in the Venn-diagram above (4). This study strategy could be used to increase our ecological understanding of many poorly understood invertebrate groups such as collembolan insects, nematodes and Tardigrae above (5 - from BBC wildlife magazine) which can be seen in this colour enhanced image to be even smaller and therefore intimately associated with hyphae (coloured green) than cecid larvae, and which, like collembolans and nematodes, dwell in the emergent cavities of decomposing plants.

brought about by the ability of paedogenic cecids to divide into daughters of much smaller size. Instead of out-running the predator, paedogenic larvae may simply enter cavity-sizes that exclude predators via thigmotropism (Wyatt 2000) - a behavioural preference for the sense of touch around the body. This explains how individual cecids, examined out of context, might seem poorly physically adapted to avoid predation whilst, at the scale of the clonal population inside rotting wood, they succeed very well in avoiding predation with asexual paedogenic reproduction and continuous movement into tight cavities at boundaries between mycelia. Perhaps the suggestion made by Keeling and Rand that spatial mechanisms can alter sexual reproductive skews, may also work in selecting for such asexual reproduction (Keeling & Rand 1995).

Increasing the extent and duration of enhanced fungal interactivity may also provide increased hyphal nourishment to cecid larvae. This would affect cecid larval life-cycles by decreasing generation time, and may also reduce environmental oxidative stress, leading to bigger cecids with more offspring which fit into cavity-size ranges appropriate to their size. Perhaps the simplest mechanism for the increase in larval number with size of mother could be the expansion and enhancement of a follicle stimulation signal as mothers increase in size (Matuzewski & Zagrodinska 1994, Chen 1970, Camenzind 1982). Small mothers, which develop more slowly (Wyatt 1960 c) give birth to fewer offspring and a greater proportion of embryos senesce and die (Akimoto 1998, Hunt 1996, Ulrich, Petals & Camenzind 1972, Camenzind 1982). Perhaps this is due to accumulative oxidative stress from feeding in mycelia with much oxidative stress and reactive oxygen intermediates (ROI), (Feder, Berlocher & Opp 1998). Some fungal metabolites have been shown to regulate genes in animals (Stadler *et al.* 1998 a, b) and contribute to phenotypic plasticity by acting as signals (Ebert 1994).

PSP zones may be refuges away from such fungal metabolic stress. It seems from the time-lapse film (Appendix 3), that mothers seek out PSP zones within which to become hemi-pupae or resting mothers (Wyatt 1967). A combination of two factors may have selected for this behaviour. The first is that if the mother becomes increasingly stressed with developing embryos inside her, she may seek out areas of less oxidative stress. The second are the combined adaptive benefits to her daughters of being born on PSP zones with less oxidative stress, more cavity complexity for thigmotropic dispersal, and accessibility to a more varied mycelial diet from two interactive mycelia.

I speculate that under conditions of drought, fungal hyphae lack the activity and protoplasmic streaming which provide nutriment to cecid methods of feeding. Cecids therefore have little adaptive choice but to enter a quiescent phase as resting mothers full of dormant larvae at the same time that fungal mycelia on either side of PSP zones become less active. In other words, cecid larvae retreat to PSP oxidative stress refuges and insulate themselves within thicker hemi-pupal cuticles with large fat reserves from which water can be produced during β-oxidation (Hunt 1996, Anderson & Beardall 1991). PSP zone cavities provide a waterproof, protective skin around quiescent larvae much like gall tissue, wax honeycomb or pupal cuticle.

An intriguing possibility is that within PSP zones cecids utilise fungal glyoxisomes (types of peroxisomes found only in plants and fungi) in order to break down lipids and thus release water and oxygen via the break down of H_2O_2 by catalase. The cecids might achieve this by piercing hyphae whilst they are feeding, allowing the protoplasmic contents of hyphae, including glyoxisomes, to enter the inter-hyphal environment. Here, lipids released in paedogenic birth (Nicolei 1961) may become acted on by fungal glyoxisomes, and

the cecids suck up the resultant sugars (Springer 1915). The cecids gain from a double production of water firstly by *H. fuscum* catalases as they degrade *V. commedens* produced H_2O_2, secondly from *H. fuscum* catalases as they act on maternal lipids released at paedogenic birth. Fungal glyoxisomes may become active on larval lipids by turning them into sugar and releasing water and oxygen (chapter 5) at the same time. PSP zones become at once refuges from oxidative stress, wetter and more gaseous environments in which cecid larvae can adjust their development as described.

The final overlapping quadrant of the Venn diagram on figure 8.4 is temperature. No evidence was successfully accumulated that fungi actually heat up woody debris in the middle of winter or that cecids affect this, though there was an interesting insignificant trend towards non-freezing temperatures deep within woody debris during frosty spells. However, temperature probe measurements showed a profound heterogeneity of hot and cold spots in the field site. Such heterogeneity was apparent during very hot and very cold periods (Worthen, Bloodworth & Hobbs 1995). There remain possibilities, on consideration of the black pigments' propensity to absorb heat energy, that PSP zones could act as reservoirs for heat storage during cold periods and also channels for ventilation of heat during hot periods through being more gaseous and cavitied. Temperature could be one way in which cecids are attracted to PSP zones, but what about secondary compounds? It has been suggested that insects can be cloaked or camouflaged from predation by the plant secondary compounds they ingest during herbivory (Stachowicz & Hay 1999). Perhaps similar effects could occur with mycophagy. It is thought that insects that become tolerant of a plant secondary compound soon adapt by using the olfactory signals provided by the chemical to guide them towards their host plant. The same could be true for cecids and the liquorice smell emitted from interactive mycelia on either side of PSP zones (Berenbaum & Zangerl 1998, Beli 1981).

The seven factors considered within the Venn diagram in figure 8.4 thus help us to understand the field distributions of cecid larvae as observed in chapter 3. Such an array of multiple causal factors (Cournot 1861, Bascompte & Sole 1995) may be involved in the distribution of many other organisms. For example, other dipteran mycophagous insects such as the Mycetophilidae, and Collembola (Setala, Marshall & Trofymov 1995), mycophagous mites and tiny tardigrades (BBC 1999) which live inside their own smaller scaled emergent cavity architectures (Hopkin 1999), and within PSP zones of many species of interacting fungi (Rukke & Midtgaard 1998), may also be affected in similar ways.

Links between scales and expansive niche surfaces provided by fungi can reveal something of importance regarding conservation (Arnolds 1990, 1997). If we are to do more than watch the increasingly isolated units of evolution dwindle (Harris, Silva-Lopez 1992, Hafernik 1992), we must appreciate the evolutionary expansion of diversity from the perspective of scale, its heterozygous boundaries and environmental interplays (Levin 1999, Kellman, Tackaberry & Rig 1998, Keitt & Johnson 1995). Perhaps there should be less focus on the individual ex-situ units of biodiversity and more on integrity of in-situ interrelationships. By lessening our disruption of the interplay throughout diverse ecological fabrics (Shiva 1994), living surfaces could be given the capacity to continue to proliferate and increase their heterogeneity (Levin 1999, Wilson 1992 a). The linking of the issue of scale and body size to causal networks (Cournot 1861, Geier 1965) relates the ecological ambits of species to fractal measures of habitat heterogeneity (Schmid 2000). This is especially true regarding biodiversity and conservation (Royama 1997). The link between heterogeneity and interactivity is important. One way of measuring habitat heterogeneity (Baker 1999) is

through fractal dimension (Ramsdale & Rayner 1997). The more space-filling efficiency a pattern possesses, the more boundary is proliferated, enfolded, branched and anastomosed within a space (Rayner 2000), and the higher the fractal dimension (chapter 2). Figure 8.5 re-iterates this point with relevance to high insect biodiversity, especially within tropical forests. In tropical forests, where insect and fungal biodiversity are possibly at their zenith, the amount of wood with potential to form cavities at fungal interactive zones, which accommodates larvae next to mycelial and wood food sources, could be phenomenal. The extent of such synergistic potential may also relate to the size of the organisms being accommodated within woody cavities.

Many of the patterns described in this project, which lead towards accommodation of insect biodiversity, may also be occurring in canopy layers (Moffett 1993) where investigation is possible with aid of tree-climbing equipment. However, the processes which seed these interactive patterns also organise themselves at much smaller scales, for example the tessellation of lichens on a bare coastal rock, or biofilm formation processes with bacteria (Rainey 1999 a,b, Ben-Jacob 1997, 1998).

Describing how chemical and physical potentials for feedback-produced pattern effects emergence and its affinity for non-linearity allows us to understand how things come into being through relation (Prigogine & Stengers 1984, Prigogine 1980). It seems highly likely that chemical and physical relations, and therefore emergence, are as much harnessed by living processes as atoms and energy are (Thom 1972, Rayner 2000, Rainey 2000, Dhamala & Lai 1999). A neat acronym for this process could be **"SSOSE" - "Synergistic seeding of synergetic emergence"** which distinguishes between synergistic and synergetic using Haken's definitions of these terms (Haken 1980). Here **"synergistic"** refers to interactive **synergistic chemical potentials** for new reactives to appear which were not in either of the original reactants (Haken 1980). The term **"synergetic"** refers to physical thermodynamic self-organisation (Haken 1980, Kauffman 1993, Ball 1999) through self-assembly and **synergetic emergence of new physical structures.**

The general process of organisation, in this case study of cecids and wood-decay fungi, is the reciprocity of exchange during relationship which leads directly to an enhanced synergistic chemical potential, and indirectly to an enhanced physical synergetic potential outcome (Bascompte & Sole 1994, 1995, Haken 1980, Sole & Bascompte 1998). In figure 8.6, examples from both abiotic and the biotic world are shown to illustrate the process whereby chemico-physical interactivity (Selander, Clark & Whittam 1991, Harborne 1988) produces asymmetric branching patterns (Chernikov *et al.* 1989). Whilst themselves not necessarily being biological (i.e. not being a direct sign of life), these patterns are as much to do with life as the living examples of patterns produced by organisms (Seifert 1992). This reflects the likelihood that life has incorporated thermodynamic chemo-physico potentials in its reactive interfaces (Baskin & Norde 2000). It seems simpler to view chemical and physical synergetics (Thorn 1998, Thom 1992, Haken 1980) as being universally present in living and non-living systems alike, than to ignore the significance of chemistry and physics in pattern-forming processes of the living world (Favre *et al.* 1988). This adds a layer of freedom to the classic deterministic genetic mechanism (Hook 1958, Rose 1997) based on the philosophical notion of mechanistic control. Life, in its most free, versatile, adaptive, explorative and error-making state (where new patterns continuously emerge from location-specific thermodynamic physico-chemical interactions) is probably liberated from direct mechanistic control but also held in check by boundary limits to deterministic chaos through indirect effects indicated by patterns with high symmetry-breakage rates and high fractal dimensions (Ruan 1998, Rainey 1999 b,c, Budd & Keating 1999, Goodwin 1994 a, b, Bascompte & Sole

Figure 8.5 The fractal significance of forest surface area, with many small-sized organisms and much dead wood. Each layer o f bark, wood, cuticle or hyphal and insect surface acts, by analogy, as a catalytic surface, regulating the exchange of energy between co-existent **interactives**, such that relationships tend to be more long lasting, and thereby influence the types of relations between ecosystem components. It is pertinent to note, regarding the enigma surrounding the magnitude of insect biodiversity, that the greatest of it's proportions live, at one time or another, or on inside the shelter provided by dead wood and its emergent architectures. This architecture, the product of the mutually attuned developmental inter-plays between fungi, insects, other invertebrates and bacteria, within the changing chemical and physical properties of the wood itself, is able to accommodate different species inside a fractal structure such that they co-exist when under normal circumstances we might expect competitive exclusion.

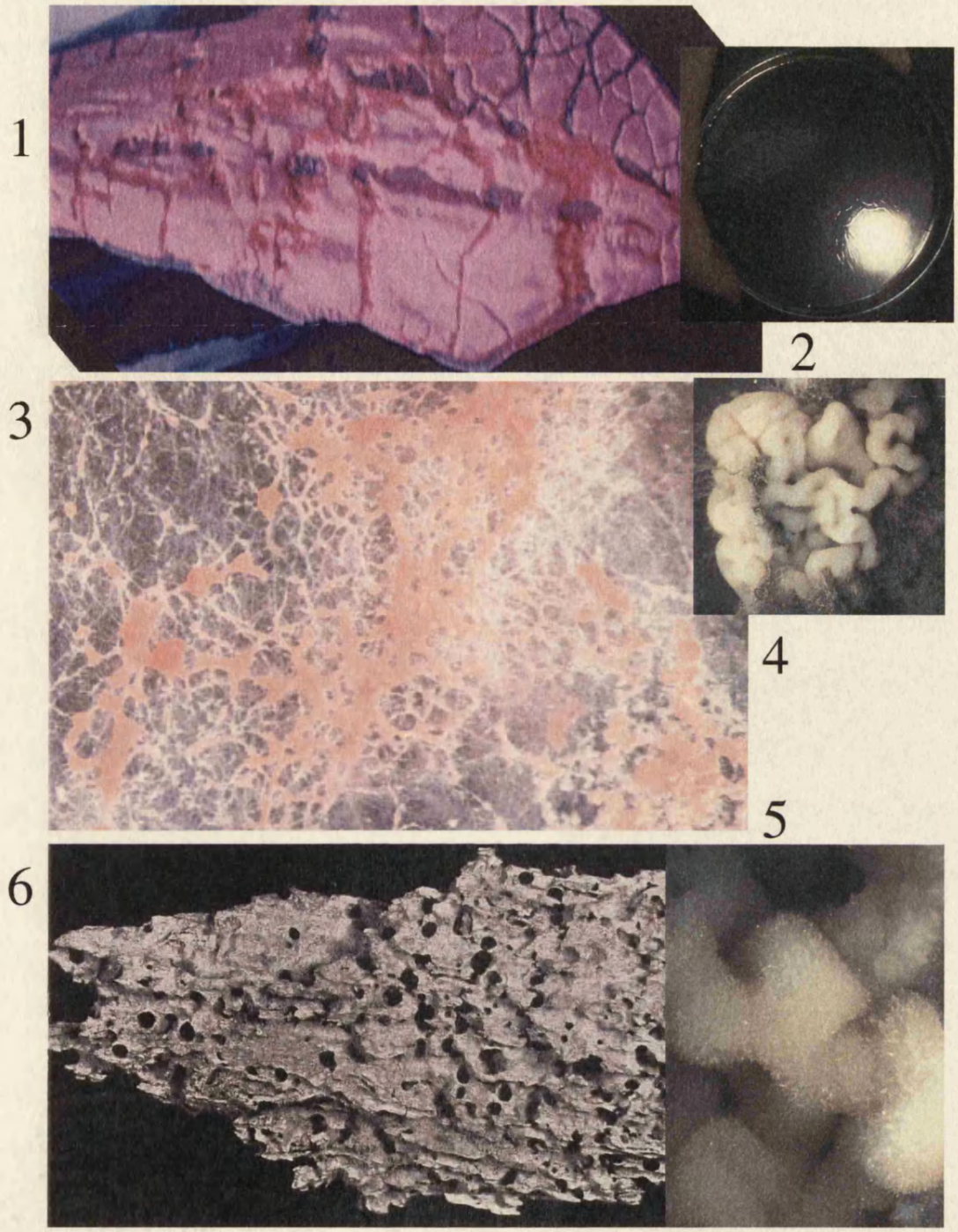

Figure 8.6 The extent to which the process of feedback across boundaries is seeded by physical and chemical potentials, outside the scope of pure biological discipline, lends a principle component to pattern development in all living systems. In the abiotic, such local feedback leads to changes in the visco-elastic properties of a party-balloon weathered to a painted post (1) which allows for the potential for branching structure to emerge. (2) the more rapid loss of water from the surface than the base of a sterile agar dish results in the emergence of an indeterminate branching,rippling pattern which increases the shape's surface area. In the biotic, the same processes are harnessed in the unique development of synergetic mycelial-yeast colony networks (3 and 4), where an additional degree of feedback between biota allows the yeast to grow outwards around hyphal branches, thereby greatly increasing hyphal branching form (5). The result of feedback between physical, chemical and several taxa combined allows for synergetic development of decompositional architecture (6); a beautiful home and refuge for countless species in the forests which cover our home planet.

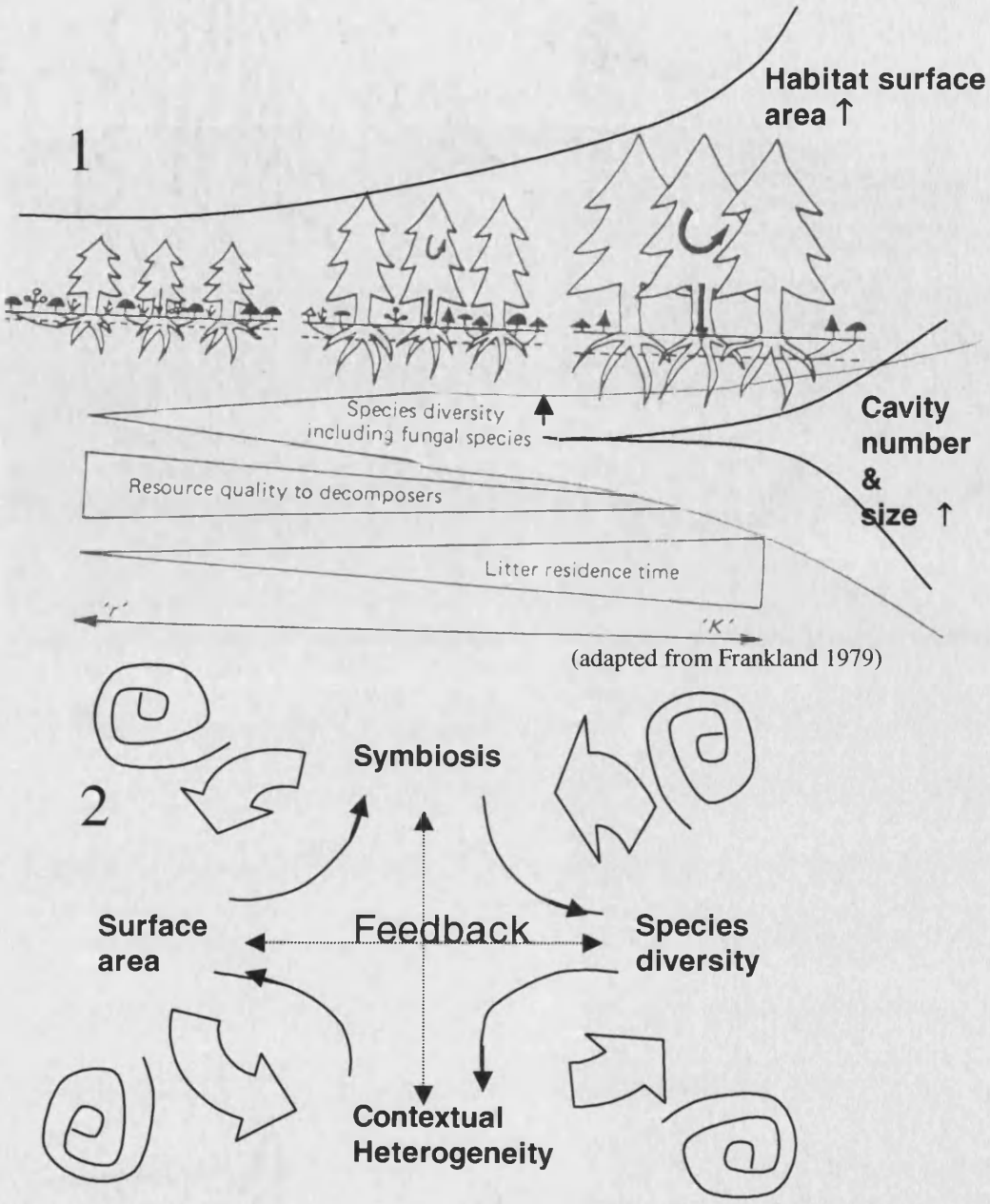

Figure 8.7 The formation of niche surface for species to inhabit involves both succession and feedback processes. (1) shows how the ability for forests to accommodate species diversity increases with forest scale and fractal architecture, involving both ecological anabolism and decomposition to create cavities.

(2) shows four macro-ecological phenomena which enhance themselves through the process of feedback. These are **symbiosis**, generating **surface area**, generating **heterogeneity**, generating **species diversity** through adaptation, generating yet more opportunity for interaction and symbiosis. If the type of symbiosis were mutual tending to be a longer-lived then the generation of new surface area would be further enhanced. The 4 factors can be linked through feedback in any order (dashed arrows), since between each factor feedback may occur positively (thick open arrows), negatively (thin line arrows) or chaotically (spirals).

1995, Rose, Kamin & Lewontin 1984). Such a process enables expansion under contraction. This gives rise to emergent patterns that maintain states of low entropy whilst receiving inputs of potentially dangerous energy. Thus energy inputs can be channelled into development and away from causing oxidative damage. Evolution perhaps works with the interactive statistical outliers from overlapping populations and their production of new synergetic forms that allow living systems to survive environmental challenge. Biological systems are possibly neither determined by linear causal event sequences nor controlled by genes. Instead, a simpler explanation is that the biological world could be sensitive to environmental processes at all scales in relation to the present and the past. In this way, if causality were described, it would be a causal and adaptive network or Gestalt (Cournot 1861). The process of coupled matching (information feedback) between the past environment (RNA + DNA) and present environment (protein, lipids, water + thermodynamics) seems to produce specific combinations of responses to particular conditions (Rainey 1999) which may be adaptive and so survive, seeded by self-organised emergent and potentially non-linear and heterogeneous patterns through indirect feedback processes (King & Schaffer 1999, Prigogine 1980, Thom 1972).

In the study of animals that inhabit dead wood, an important point to highlight from this project is a non-random size class cavity allocation, or "**If you want to find an organism, look for it in the right sized hole**". What could the implications of this be for biodiversity conservation? It is known that to conserve some flagship species, for example Canadian flying squirrels, salamanders and large woodpeckers, provision of artificial dead trees with appropriately scaled cavities (aka nesting boxes) is very important (Okland 1994, 1999, Noss 1992, Pickett, Parker & Fielder 1992, Novotny & Kindlman 1996). Either cavities are provided artificially or a reduced removal of dead wood has to occur. The implications of this strategy can now perhaps be more fully appreciated. Only the largest woody debris may possess a physical potential to form large enough cavities to accommodate large flagship species (Collins & Thomas 1991, Harris & Silva-Lopez 1992). Not surprisingly there is the correlation between size of woody debris and potential cavity-size classes which may emerge. Also the range of cavity-sizes increases with size of woody debris (chapter 3). Woody debris from longer-lived (Irmler, Heller & Warning 1996) more K-selected tree species has been shown in chapter 3 to have a physical potential for a greater size range of cavities than more r-selected pioneer species (Hedge, Chandron & Gadgil 1998). This relates to a distal explanation that endemic organisms like the English oak *Quercus robur*, which have the largest numbers of symbiotic co-existent relations, have predominated in particular regions for the longest periods of evolutionary time, and thus had more potential for co-existent relationships to become commensal and mutual partnerships (Sevenster 1996). *Quercus robur* is not only a large tree species, but also is one of our most long-lived, from both ecological and geographical perspectives. Taken together with the theory of synergistic interactivity that this project promotes, these two theories can now help to explain how *Quercus robur* provides more accommodation for more different types of invertebrate than any other plant species in the British Isles. In this sense, the structure of the tree provides active sites as a catalyst for the interactive co-existences that increase biodiversity. The larger and more ancient the tree species, the greater range of cavity-size classes the tree's decomposition will present as available for invertebrate accommodation. In the tropics this also holds true for accommodation of vertebrates. This is a theme that will be returned to later.

For now, let us change scales briefly to consider parallel trends of boundaries and cavities and their selective catalytic ability in microcosm. A given hole in a boundary may block the passage of a substance or let it through according to the size of that substance (Forman 1995). However, from the standpoint of the

substance, a membrane with holes large enough for the substance to freely flow through simply does not exist. The presence of a membrane is relative to the substance it blocks. For example, as far as a molecule of water is concerned, there are many boundaries within an organism that simply do not exist. Only those boundaries that are external, or part of a distributive system, are insulated so as to block the passage of water. For any exchangeable substances, the presence or absence of a boundary depends on the action, or not, of a gate or other dynamic permeability/porosity control (Haken 1980, Baskin & Norde 2000). There thus exist, in life systems, a nested hierarchy of scales relative both to substance size and boundary pore size (Forman 1995). A substance of certain size may have its movement blocked by a network of many internal boundaries at one instance, thus being confined to localised activities, and blocked by very few in the next, at which times it is free to move via osmosis and diffusion kinetics (Einstein 1926) until being blocked by the next boundary. It is at boundaries that reactions occur more frequently than with freely diffusing reactives. The symbiosis between cecids and wood-decay fungi is also concerned with the acquisition of substances of various sizes through the interaction of dynamic boundaries between one organism and another. The research has shown that the principles of semipermeability and thermodynamics can be applied to ecology, and that ecological ideas can be applied at all scales from bio-chemical interactions of protein populations, metabolic feedback loops, across membranes and within cytoplasm and between scales of ecological assemblage from molecules to populations of trees, fungi and resident insects.

Fungi provide a fundamental dynamic to forest architecture, niche space and surface area. Figure 8.7 shows the rise in biodiversity with the rise in cavity formation and boundary surface area. Also figure 8.7 suggests that four macro-ecological processes of symbiosis and co-existence, generation of new surface area, generation of habitat heterogeneity (Tokeshi 1999) and rise in synergistic diversity (at any scale), all involve pattern-forming processes of reciprocal developmental feedback (Haken 1980, Mees 1981, Baskin Norde 2000, Brasier 1995, 1999, Rayner & Coates 1987, Atkinson & Shorrocks 1984). Without such dynamics it is unlikely that forests could exist let alone support a rich variety of species (Dennis 1997, Wertheim *et al.* 2000), or that such a rich diversity of life forms could have made itself available to the process of natural selection (Darwin 1859). Evolution perhaps can also be seen as a feedback system between an organism's phenotype and information signals relating to the environment which are inclusive with it, producing changes, and the divergence of populations into new species, over hundreds of thousands of divergent life-cycles (Ho 1993, 1995 b, Rainey 2000, Rayner 2000, Tokeshi 1999).

Returning once more to the conservation theme, the above micro-macro synthesis of developmental feedback may teach us something about how to sustain biodiversification as a process. To enable biodiversity to flourish we need to shift our perspective of conservation so that it now encompasses the dynamics that enable heterogeneity to grow in an ecosystem. We could possibly achieve this by redoubling our efforts to maintain and enhance habitat surface area within an ecosystem (Waring 1982). This could be achieved by leaving dead and fallen wood to decompose whenever possible, to become colonised by the huge numbers of small species that have co-evolved with fungi during wood-decay processes. We could also conserve the processes that allow trees to self-seed, and thereby replace large mature cavities over time with small newly forming ones, during a woodland succession and re-forestation process. To do this may require efforts to counter the popular misconception that dead wood is messy and needs to be cleared. More crucially, an ecological message needs to be heard of the unique opportunities provided by any piece of dead wood left to lie as it falls, through provision of networked heterogeneous habitable space. Asymmetric networks of

habitable space, nested across spatially fragmented and patchy resources, make the difference between a habitat being one in which a particular species can thrive (source) or suffer a gradual local extinction (sink) (LoFarro & Gomulkiewicz 1999). It is likely that forest architectures that have the highest surface area possess the highest heterogeneity, greatest efficiency in filling space, and highest fractal dimensions (Close 2000, Pradham 1964, Goldberg & Novoplansky 1997, Dennis, Young & Gordon 1998, Casper, Cahil & Jackson 1999). Such structures also exhibit the most highly branched symmetry (Hanke & Green 1994, Kooi, Boer & Kooiman 1998, Petukhov 1989). Moreover, we need to leave behind the cultural baggage that we often impose on nature, that symmetry of pattern represents a greater status in evolutionary achievement than broken symmetry. While symmetrical forms are efficient and rapid in expanding and proliferating, they may be inefficient at filling space and conserving. Conversely where asymmetric forms are efficient in filling space and forming conservation networks, they may be inefficient in expanding and proliferating. Asymmetric forms like these are exemplified by forests, reefs, lichens and biofilms. They are full of variety of surface areas, cavity-sizes and shapes, and consequently accommodate a greater number of different co-existing, niches and interactive species (Krijger, Svenster 1999, Kuyper 1998, Rainey & Travissano 1998, Stoodley, Boyle, Dodds & Lappin-Scott 1997, Veron 2000, Bennet 1997, Ben-Jacob 1997, 1998, Wilson 1992 a, Rodenhouse, Sherry & Holmes 1997). Such emergent architecture (Ball 1999), through requiring the longest time to emerge (due to its topological manifoldedness), is highly efficient in allowing organisms to fill space, provides structure which reduces competition between symbiotic co-inhabitants, and tends towards less competitive relations (Rainey 2000, Rayner 2000, Levin 1999, Ball 1999, Axelrod 1984, Hamilton & Stickler 1993, Santos, Eisses & Fontevila 1999, Dierderich 1996, Etges 1998, Frank & Amarasekare 1998, Erwin 2000, Grimaldi 1985, Parsons 1996, Murtagh 1998, Dover & Flavell 1982).

8.3.2 Putting this work in context

8.3.2.1 Overall Summary

As far as I am aware, this project has been the **first to investigate reciprocal developmental interactivity between insects and fungal inhabitants of decomposing wood.** Also this project is the **first to examine inter-specific interactions between saprotrophic fungi of different sub-divisions** at **both field and metabolic scales**. Previous reviews have highlighted the need for such work in saproxylic systems (Swift & Boddy 1984, Dowding 1984, Dajos 2000). Because of the nature of the project and the fact that it necessarily broke new ground, **12 new experimental techniques** were developed and tested during the tenure of study.

8.3.2.1.1 Five new fieldwork methods

This project developed new fieldwork methods: **(1)** a new protocol for rapid macro-fungal biodiversity surveys with compared Margalef indexes (chapter 2). As far as I know, no such method has been developed for the fungal kingdom or integrated into functional studies to date (Taylor 1993, 1994), although similar methods existed for birds, mammals, insects, amphibians, trees, ground and epiphytic vegetation (Moffett 1993, Wilson 1992 a, Dajos 2000, Cannon 1996, 1998). **(2)** Integration of this new rapid fungal biodiversity survey together with a nested survey of fractal dimensions enabled an assessment of habitat heterogeneity at different scales for the assemblage of dead wood fungal communities and insects which inhabit them (chapter 3). **(3)** This enabled the rest of the project to be designed around an integrated investigation of multiple-scales at which interactions between insects and fungi were observed to proceed. As far as I know,

single items of small woody debris of less than 10 cm diameter have not been used for emergence trapping **(4)** for inhabiting insects, and *Corylus avellana* wood has not been used for this purpose before (Dajos 2000). Emergence trapping has occurred from whole woodpiles and associated soil before, from large basidiomycete fruiting bodies, and from large fallen trunks of Douglas fir (Dajos 2000, Owen 1992, 1989). **(5)** The method to subject cecids to trials of height and distance "jumped" is also new and, as far as I know, data on such behaviour has never been obtained before.

8.3.2.1.2 Seven new laboratory methods

This project developed novel laboratory methods. These were the first PSP-productive interactive experiments **(1)** to be carried out between ascomycete and basidiomycete mycelia. Bipartite fungal research had not investigated PSP-producing metabolic pathways between such different fungal groups before. Previous work on intra-specific somatic incompatibility was carried out in ascomycete species (Crowe 1997, 1998) and in Basidiomycetes (Watkins 1998, Watkins, Beeching, Rayner 1998 a, b), whilst inter-specific investigations occurred only between fungi of the same kingdom sub-divisions (either within the Basidiomycotina or Ascomycotina). As far as I know, this was the first work examining in detail the metabolism of PSP-producing interactions between sub-divisions of the fungal kingdom. Moreover, what makes this work particularly novel is that this detailed investigation at field and metabolic scales proceeded in concert with treatments of developing mycelia with mycophagous cecid larvae, and compared responses of mycelia to insects with and without mycelial inter sub-division interactivity. Where metabolic and developmental interactive investigations proceeded in culture, six novel tissue-lysis printing protocols for total protein **(2)**, antioxidants **(3)**, free radicals **(4)**, hydrogen peroxide **(5)**, peroxidase **(6)** and catalase activity **(7)** were developed for use in concert with HPLC and TLC chromatography (chapters 4 -6). Such work, as far as I am aware, is unprecedented to date.

8.3.2.1.3 Seven major findings overall

There were seven principal findings of this project overall. **Firstly** was the discovery of the importance of PSP zones in the field distribution of the mycophagous cecid *B. fraxinicola.* **Secondly** the presence of *B. fraxinicola* larvae on PSP-producing interactive mycelia enhanced metabolic oxidative feedback between the ascomycete and basidiomycete components of the system, so as to increase the available quantity of PSP. **Thirdly** metabolic feedback processes enhanced by cecid larval treatments increased the physical potential for cavitation and bark loosening associated with PSP chemistry. **Fourthly** the enhancement of physical space architecture via chemical mediation between interactive mycelia was identified as a component of the schema termed SSOSE (Synergistic seeding of synergetic emergence). **Fifthly,** the reciprocal enhancement of heterogeneity which is brought about by interaction, and the resultant increased co-existence of *Vuilleminia commedens* and *Hypoxylon fuscum* through increased mycelial PSP boundary thickness, suggested a role of interactions in catalysing the coupling processes which generate heterogeneity, new surface area, symbiosis and biodiversity in a feedback cycles of ecological anabolism. This interactionist view of ecological assemblage beares some resemblance to the views expressed by Clements in 1917 (Maurer 1999). **Sixthly** the cavitation of dead wood into complex arrays of micro-tree holes and rot-holes was linked to their suggested vital roles in accommodating much insect and invertebrate forest life (Dajos 2000, Moffett 1993). Finally, **seventhly,** the effects of insects and fungi on nutrient cycling processes and on decay rate remain unclear, depending on how these are measured and at which scales (Swift & Boddy 1984, Dowding 1984). Work here has suggested that, when considering nutrient cycling, future work must

also consider the physical scale (in terms of distance) and rate of cycling (in terms of times to complete) as well as measured amounts of nutrient ingredients themselves. When considering decay rate, work in future should consider the scale at which gross measurements, such as of wood density and hardness, have meaning to the study organisms and whether measurements are relevant to the suggested durability of fragile and intricate PSP architectures (Swift & Boddy 1984, Dowding 1984).

8.3.2.1.4 Eleven contextual themes

Themes that became incorporated into the conceptual part of this project were as follows. The importance of **symmetry**-breaking **(1)** and maintenance to **development** strategies **(2)** of different taxa. **Synergy** induced by interactive relations **(3).** Chemical and physical **seeding** of morphogenic emergence **(4). Fractal dimension (5)** as related to heterogeneity, manifolded symmetry, space-filling distributions of organisms in forest ecosystems. **(6)** r-K-selected **successions** of decomposer fungi and associated biodiversity. **(7)** niche-space as refuge from **oxidative stress** as well as predation. **(8)** *B. fraxinicola's* highly **correlated aggregation** to the structure of *H. fuscum's* **PSP** zone **(9).** Topological processes that enhance **co-existence (10)** via chemical oxidative and water-stress alleviation, catalysis and **developmental feedback (11).**

Figure 8.8 shows a schema for reciprocal interaction in the *Brittenia-Hypoxylon*-PSP-*Vuilleminia-Corylus* pentapartite system in which there are 20 different interaction effects to consider. Over the page, table 8.1 describes these 20 effects in light of work carried out in this project.

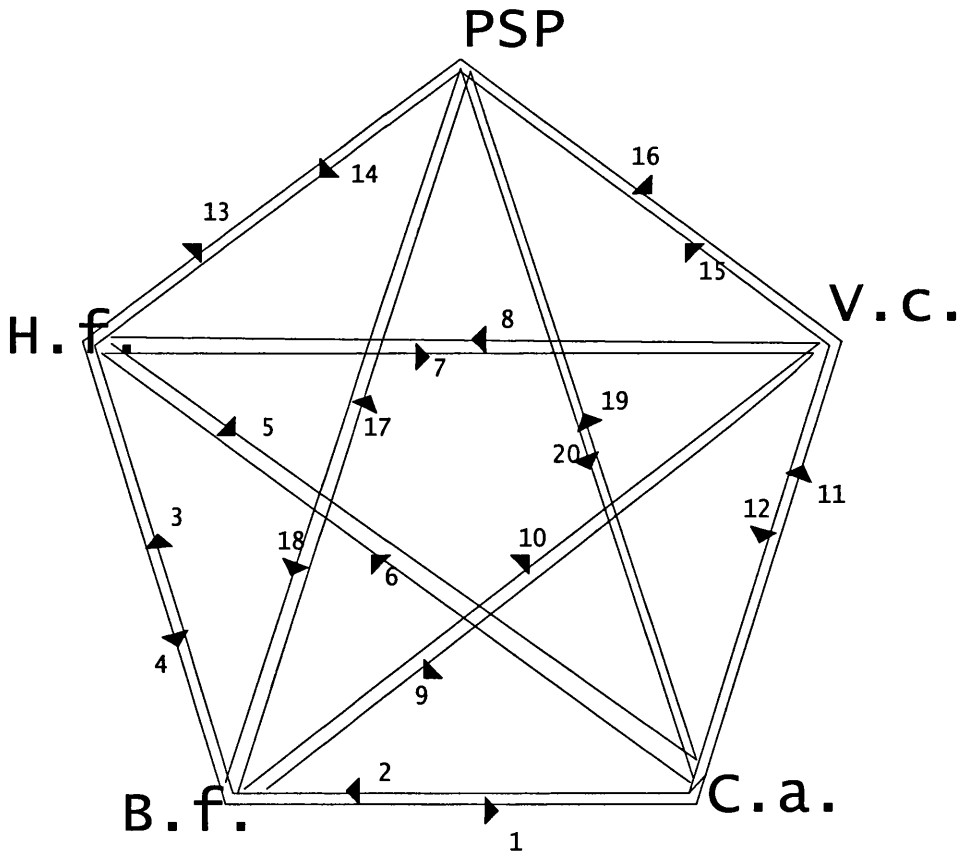

Figure 8.8
The *Brittenia-Hypoxylon*-PSP-*Vuilleminia-Corylus* system: a pentapartite system with 20 directions of effect. **B.f** = *Brittenia fraxinicola* cecids, **H. f.** = Ascomycete *Hypoxylon fuscum*, **V.c.** = Basidiomycete *Vuilleminia commedens*, **C.a.**= *Corylus avellana* hazel, **PSP** = pesudosclerotial plate.

TABLE 8.1 ***Brittenia-Hypoxylon*-PSP-*Vuilleminia-Corylus*** **- a pentapartite system of 20 effect directions (see fig. 8.8) Key: V.c.=** *Vuilleminia commedens*, **H.f.=** *Hypoxylon fuscum*, **PSP** = pseudosclerotial plate, ***Corylus*** = hazel, **Cecid =** *Brittenia fraxinicola* larvae.

Effect no.	effect: (A on B)	Description of the effect (e.g. of Cecids on *Hypoxylon fuscum*)
1	Cecid on *Corylus*	Enhanced decompositional cavitation: indirect via fungi, direct via growth pressure.
2	*Corylus* on cecid	Provision of initial conditions, thickness of bark, inter-medullary ray distances, latent fungi, direct effects.
3	Cecid on H.f.	Facultative mutual ecto-symbiosis, increase in PSP, antioxidants and H_2O_2, protection of Hf from Vc invasion.
4	H.f. on cecid	Facultative mutual ecto-symbiosis, mycelium - food & oxidative stress, PSP - stress-free & no food. Alteration of paedogenic Gno/Gt ratio.
5	H.f. on *Corylus*	Softens bark matrix, bark loosening, cavity architcture development, unlocks nutrients. Increases heterogeneity.
6	*Corylus* on H.f.	Lignin nutritive source, initial conditions of bark thickness, woody debris girth. Provision of high "catalytic" surface area. Bark-protection/ insulation from dessication & frost.
7	H.f. on V.c.	PSP development, H_2O_2 level increased loss and gain of polar and non-polar metabolites.
8	V.c. on H.f.	PSP development, antioxidant levels increased, loss and gain of polar and non-polar metabolites.
9	V.c. on cecid	Provision of food and oxidative stress, H_2O_2, ROI, free radicals. Alteration of Gno/Gt ratio.
10	Cecid on V.c.	Increased levels of H_2O_2, ROI, reduced combative ability, increased aerial mycelium underwhich cecids can be insulated from stress.
11	*Corylus* on V.c.	Lignin nutritive source, initial conditions of bark thickness, woody debris girth. Provision of high "catalytic" surface area. Bark-protection/ insulation from dessication & frost.
12	V.c. on *Corylus*	Softening and cavitation of bark matrix, bark peeling, unlocks nutrients, increases heterogeneity.
13	H.f. on PSP	PSP production through free radicals, melanisation of ROI-antioxidant complexes; thereby sequestering free radicals and providing an oxidative-stress-free zone.
14	PSP on H.f.	Protective hydrophobic collective hyphal boundary against antagonistic mycelia of other species.
15	V.c. on PSP	PSP synergies, site-dependent, contribution of ROI which provide energy for PSP matrix polymerisation.
16	PSP on V.c.	Prevention of invasive fans and chords, reducing invasive capacity of Vc
17	Cecid on PSP	Enhanced PSP production via perturbation in fungal metabolism.
18	PSP on cecid	Provision of stable cavity matrix oxidative stress free refuge into which larvae can ramify.
19	*Corylus* on PSP	Initial conditions e.g. bark thickness, variability in potential for combined abiotic flux and metabolisms to enhance bark-loosening process.
20	PSP on *Corylus*	Persistent decompositional hydrophobic structures whilst rest of wood rots away, revealing a matrix of cavities.

8.3.2.2 Comparison to other work

There are 15 overlapping areas of fungal-insect and insect/invertebrate biology (Table 8.2) whose authors' findings and suggestions can be compared and contrasted with those emerging from this thesis.

TABLE 8.2: Invertebrate/Insect-fungal literature:

AREA OF WORK	AUTHORS	Partism
Mycophagous *Drosophila* population ecology and genetics	(Shorrocks *et al.* 1984), (Courtney, Kibbota & Singleton 1990), (Lacey 1984), (Hanski 1989, 1998, 1999), (Grimaldi 1985), (Nunney 1990), (Toda, Kimura & Tuno 1999), (Shorrocks & Charlesworth 1982), (Abrams 1999), (Santos, Eisses & Fontdevita 1999), (Grimaldi & Jaenike 1984), (Worthen & Honey 1999), (Shorrocks & Bingly 1994), (Shorrocks *et al.* 1991), (Ashe 1984), (Toda & Kimura 1997), (Montague & Jaenike 1985), (Jaenike & James 1991), (Powell & Taylor 1979), (Roos & Sabelis 1995), (Sih 1997), (Worral 1999), (Pelletier 2000), (Worthen, Carswell & Kelly 1996), (Worthen, Bloodworth & Hobbs 1995), (Hodin & Riddiford 2000 b), (Hodin 2000), (Delpuech *et al.* 1995)	Mono Bi
Mycophagous, + paedogenic cecids + fungi	(Darwin 1959), (Kahle 1908), (Springer 1915), (Nicolei 1961), (Ulrich 1934, 1943, 1962, 1972), (Gabritchevski 1929), (Fux 1974), (Camenzind 1963, 1982), (Wyatt 1959, 1964, 1960 a,b,c, 1961, 1963 a,b, 1965, 1967, 1969, 2000), (Mamaev & Krivosheina 1993), (Hunt 1996, 1997), (Spencer 1998), (Hodin 2000), (Hodin & Riddiford 2000 a), (Herman, Hynum & Alexander 1993), (Batra & Lichtwardt 1963).	Mono
Fungi,Sciaridae and Mycetophilidae	(Boddy, Coats & Rayner 1983) (Hatcher 1995) (Swift & Boddy 1984), (Dowding 1984), (Swift, Heal & Anderson 1979), (Dajos 2000), (Delporte 1987), (Hammond & Laurence 1989), (Wertheim, Sevenster & Eijs 2000), (Sevenster 1996), (Shorrocks & Sevenster 1995), (Todd 1988), (Worthen, Bloodworth & Hobbs 1995), (Pirozynski & Hawksworth 1988)	Mono Bi
Insects + mycelial development	(Jennings & Rayner 1988), (Wessels 1993, 1998), (O'Conner 1984), (Cooke & Rayner 1984), (Swift & Boddy 1977), (Frankland, Poskitt & Howard 1995), (Moore 1996), (Martin 1992, 1987, 1979), (Hickin 1975), (Safar & Cooke 1988), (Dutton & Evans 1996), (Delporte 1997), (Rayner 1976, 1984 a,b), (Rayner & Webber 1984), (Rayner 1997), (Cooke & Rayner 1984), (Morin 1999), (Hatcher 1997), (Kahle 1908), (Cooke 1977), (Bruns 1984), (Davidson *et al.* 1996), (Ives 1988)	Mono Bi
Insects from agarics + polypore brackets and other fruiting bodies	(Dajos 2000), (Guevara, Rayner & Reynolds 2000), (Wertheim *et al.* 2000), (Gilbertson 1984), (Guevara & Dirzo 1999), (Kimbrough 1984), (Grimaldi 1985), (Lacy 1984), (Hammond & Laurence 1989), (Dowding 1984), (Swift, Heal & Anderson 1979), (Worthen, Carswell & Kelly 1996), (Worthen, Mayrose & Wilson 1994), (Worthen, Bloodworth & Hobbs 1995), (Worthen & Moore 1991), (Worthen 1989 a,b) (Worthen *et al.* 1995), (Worthen & Moore 1991), (Worthen, Jones & Jetton 1998), (Blackwell 1996), (Martin 1979), (Todd 1988), (Shorrocks & Sevenster 1995), (Rodenhouse, Sherry & Holmes 1997), (LoFaro & Gomulkiewicz 1999), (Ives 1995), (Sevenster 1996), (Jaenike & James 1991), (Morin 1999), (Chandler 1990), (Dobzhansky 1970), (Laurence 2000), (O'Conner 1984), (Hatcher 1995), (Hatcher *et al.* 1994), (Bardner, Fletcher & Hamon 1984), (Dowding 1984), (Anderson, Rayner & Walton 1984), (Swift & Boddy 1984), (Pirozynski & Hawksworth 1988).	Mono Bi
Indirect effects between insects, fungi + plants	(Morin 1999), (Hatcher & Ayres 1997), (Hatcher 1995), (Worthen & Moore 1991), (deNooje, Biere & Linders 1992), (Dajos 2000), (Anderson, Rayner & Walton 1984), (Swift & Boddy 1984), (Dowding 1984), (Rabin & Pacovsky 1985), (Gange & West 1994), (Leath & Byes 1973), (Leath & Newton 1969), (Shinkaji, Okabe & Amano 1988), (Bi, Felton & Mueller 1994), (Rayner 1997), (Bonsall & Hassell 1999), (Fryar 1998).	Bi Tri
Ants and Termites	(Batra & Batra 1979), (Sands 1969), (Wood & Thomas 1989), (Dajos 2000), (Hickin 1975) (Cooke & Rayner 1984), (Pirozynski & Hawksworth 1988).	Bi
Coleoptera and fungi	(Guevara, Rayner & Reynolds 2000), (Beaver 1989), (Spence *et al.*, 1997), (Wheeler 1984), (Staniland 1930), (Webber & Gibbs 1989), (Swift & Boddy 1984), (Kimbrough 1984), (Lawrence 1973, 1977), (Hanski 1989), (Swift & Boddy 1984), (Dowding 1984), (Webber & Brasier 1984), (Webber & Gibbs 1989), (Yamoaka *et al.* 1997), (Beryman 1989), (Ingvarsson, Olson & Ericson 1997), (Pielou & Verma 1968), (Pielou & Matthewman 1966), (Paviour-Smith 1960 a,b), (Hatcher 1995), (Hertert, Miller & Partridge 1975), (Hickin 1975), (Murro 1926), (Klepzig *et al.* 1995)	Mono Bi Tri
Lepidoptera and fungi	(Rawlins 1984), (Powell 1971), (Powell & Skaley 1975), (Rabin & Pacovsky 1985), (Hickin 1975), (Hatcher 1995)	Mono Bi

Crop plants Insects & entomo-pathogenic fungi.	(Charnley 1997), (Clarkson & Charnley 1996), (Bultmen *et al.* 1998), (Bultman 1995, 1996), (Kimborough 1984), (Hatcher 1995), (Christianson & Wilcoxson 1966), (Jarvis *et al.* 1984), (Powell & Skaley 1975), (Dowd 1969), (Pirozynski & Hawksworth 1988).	Mono Bi
Theoretical biology of environmental interactions (symbiosis + heterogeneity, feedback, scale ergodic chaos, Lyapunov, refuge space, source-sink habitats, stress alleviation, cavitation, site-specificity, site-dependance, synergy, epigenetics)	(Schmid 2000), (Dajoz 2000), (Morin 1999), (Watts 1999), (Massey *et al.* 1999), (Shin & Hammond 1998), (Rodenhouse, Sherry & Holmes 1997), (Royama 1997), (Rayner & Franks 1987), (Brown 1999), (Lowman 1992, 1997), (Rayner 1997), (Couzin 1999), (Kauffman 1993), (Baker 1982), (Gauthreaux 1980), (Dirzo 1984), (Harper 1977), (Hanski 1989), (Lawton & Strong 1981), (Dennis 1997), (Whittacker & Levin 1975), (Levene 1953), (Levin 1999), (Rose 1997), (Ball 1999), (Maurer 1999), (Hunter 1999), (Whitehead 1978), (Goodwin 1994), (Margulis 1998), (Tokeshi 1999), (Hawkins, Mathew & Hockbery 1993), (Atkinson & Shorrocks 1981, 1984), (Parsons 1996), (Grimaldi & Jaenike 1984), (Shorrocks, Rosewell & Atkinson 1984), (Kitching 1971), (Danielson Muzzio & Ottino 1991), (Rainey 1993, 199 a,b,c), (Rainey & Travisano 1998), (Barton & Clark 1990), (McDonald & Kojima 1974), (McDonald, Bale & Walters 1998), (West & Deering 1995), (Hastings, Hom, Ellner, Turchin & Godfray 1993), (Turchin & Taylor 1992), (Hassel, Lawton & May 1976), (Lorenz 1985), (Wolf *et al.* 1985), (Falk, Bjornstead & Stenseth 1995), (Ellner & Turchin 1995), (Bankal & Krivan 1999), (Sutherland *et al.* 1980), (Shorrocks *et al.* 1991), (McDonnald & Kojima 1974), (Courtney, Kibota & Singleton 1990), (Jablonka & Lamb 1995), (Hatcher 1995, 1997), (Carter 1973), (Lander et.al. 1996), (Ponge 1991), (Bowers 1991), (Brooks & Wiley 1986), (Carrol 1995), (Sterelny & Griffiths 1999), (Favre *et al.* 1988), (Ho 1993 a,b), (Hodin 2000).	Bi Tri Quad Penta
Metabolic Interactivity: Synergy, 2° compounds, free radicals, oxidative stress, membranes, insulation, symmetry breaking, emergent states	(Bar-Or 2000), (Dajos 2000), (Stadler *et al.* 2001), (Salvador 2000), (Bascompte & Sole 1995), (Moller & Swaddle 1997), (Rayner 1997), (Hatcher 1995), (Hatcher & Ayres 1997), (Hatcher *et al.* 1994), (Carter 1973), (Raffra & Smalley 1995), (Bronner, Westphal & Dreyer 1991), (Kant & Ramani 1988), (Hilderbrand *et al.* 1986, 1989), (Edwards *et al.* 1991), (Sutherland *et al.* 1980), (Hashimoto & Asakawa 1998), (Whalley & Edwards 1995), (Felton, Summers & Mueller 1994), (Bi *et al.* 1994), (Williams 1970), (Cymborowski 1992), (Wigglesworth 1970), (Gillespie 1991), (Keitt & Johnson 1995), (Hunt 1997), (Roitberg & Isman 1990), (Jork, Funk & Fischer 1990), (Byung 1993), (Hammerschmidt, Nuckles & Kuc 1982), (Shimazono 1951), (Popp, Kalyanaraman & Kirk 1990), (Charudattan 1986), (Chang & Thrower 1981), (Sondheimer & Simeone 1970), (Leath & Newton 1969), (Neville 1975), (Gilmor 1965), (Koch 1994), (Brooks & Wiley 1988).	Bi Tri Quad Penta
Hymenoptera and fungi	(Dajos 2000), (Hunter 1999), (Swift & Boddy 1984), (Cooke & Rayner 1984) (Cartwright 1929), (Hickin 1975), (Gilbertson 1984).	Mono Bi
Mites and fungi	(Dajos 2000), (O'Conner 1984), (Athias & Binche 1989), (Lussenhop 1992), (Hatcher 1995), (Ydergaard, Enkegaard & Brodsgaard 1997), (Cooke & Rayner 1984), (Morin 1999), (Yeates 1987).	Mono, Bi, Tri
Wood decomposition, mycelia and insects	(Dajos 2000), (Bokova 2000), (Ehustrom 2001), (Rodgers 2000), (Tokeshi 1999), (Mamaev, Krivosheina 1993), (Rayner 1993 a,b), (Rayner 1997), (Rayner, Boddy 1988), (Rayner, Way 1999), (Swift, Boddy 1984), (Cooke, Rayner 1984), (Martin 1984), (Dowding 1984), (Hickin 1975), (Crowson 1984), (Hatcher 1995), (Pirozynski 1988), (Pirozynski, Hawksworth 1988), (Hawksworth 1991), (Irmler, Heller, Warning 1996), (Fisher 1940, 1941), (Fisher, Parkin 1930), (Becker 1938), (Temnuhin 1996).	Mono, Bi

Regarding the previous table 8.2, the following paragraphs tackle each area of work sequentially to compare and contrast with work presented here on the ***Brittenia-Hypoxylon-PSP-Vuilleminia-Corylus*** system.

8.3.2.2.1 Mycophagous Drosophila

Most fieldwork using mycophagous *Drosophila* has been carried out using agaric fruiting body baits (Shorrocks & Charlesworth 1982, Grimaldi 1985, Hanski 1989). The literature is biased towards shorter-lived agarics such as *Agaricus bisporus* baits (Lacey 1984, Worthen, Carswell & Kelly 1996), rather than longer lived crustose or pustular fruiting bodies from fungi inhabiting dead branches and hazel poles, such as *H. fuscum* and *V. commedens*. Some reference is made to occasional *Drosophila* from persistent bracket fungi (Gilbertson 1984, Dajos 2000). No work has been carried out on insects that inhabit *H. fuscum* or *V.*

commedens before. Work on niche choice (Hanski 1989) and source-sink habitats (LoFarro, Gomulkiewicz 1999), suggests some parallel to niche choice in paedogenic cecids. It is likely that the issue of habitat structural space influences niche choice for larvae, with larger sized species of insect generally having an increased propensity to be mono-phagous whilst smaller species of Drosopholidae and Cecidomyiidae tend towards polyphagy - at least in their sink habitats (Pelletier 2000, Roos & Sabelis 1995). However, *Brittenia fraxinicola* appears highly correlated (Worthen, Carswell & Kelly 1996, Ives 1988, 1995, Sevenster 1996) with its source habitat in architectually complex *H. fuscum* PSP-zones interactive with a range of other Basidiomycetes including *V. commedens*. Whilst able to survive in much lower density in sink habitats on a range of other interactive and non-interactive fungi, *B. fraxinicola* does seem to favour site characteristics provided by the ascomycete *H. fuscum* in order to produce extensive colonies. This is especially so when fungi are in interactive states. Regarding predator avoidance (Sih 1982), there appears to be a trade-off and possible "Allee" effect (Morin 1999, Speight, Hunter, Watt 1999) between food availability and refuge from stress, which in *Drosophila* tends to be associated with escape from predators, but with *B. fraxinicola*, appears to be a zone of reduced oxidative stress. Oxidative stress appears to be a cost associated with living inside a mycelium that decays lignin, as do both *H. fuscum* and *V. commedens*. A suggested "Allee" effect (Worral 1999) may also exist whereby low cecid population densities reduce reproductive success due to sink habitat characteristics such as lack of space, increased predation and lack of refuge, whilst at heavy densities, particularly large and mature source habitats, attraction of predators such as other predatory larval species, birds, parasitic wasps and competition from other saproxylic insects such as beetles may increase (Courtney, Kibota & Singleton 1990, Toda & Kimura 1997, Toda, Kimura & Tuo 1999). This situation points towards intermediate population densitiy and intermediate disturbance preferences for a population of mycophagous fly larvae to persist (Pelletier 2000, Rodenhouse, Sherry & Holmes 1997). Fungal habitats are particularly ephemeral and heterogeneous with ever-changing dynamics caused by fungal decomposition of wood and fruiting body decay (Diffendorfer 1998, Wertheim *et al.* 2000). In general **the *Brittenia-Hypoxylon*-PSP-*Vuilleminia-Corylus* system supports the notion that small mycophagous Diptera are excellent model organisms for studying the cross-over between bi and multipartite interactions with metapopulation distributive, genotype - phenotype, behavioural ecology** (Courtney, Kibota & Singleton 1990, Wertheim *et al.* 2000, Hatcher 1995, Rodenhouse, Sherry & Holmes 1997).

8.3.2.2.2 Paedogenic cecids

In 1861 when Wagner first peeled bark from rotting Russian Lime and Rowan trees and discovered a new sub-family of small flies which reproduced by paedogenesis, he had no idea of the scientific controversy this would create in St. Petersburg (Khale 1908). This fascination with paedogenesis carried over into Darwin's revised sixth edition of the Origin of Species, as it seemed to Darwin to confirm his belief in the "essential identity of the process of alternate generation and of ordinary metamorphosis" (Darwin 1859). Interestingly Darwin also mentions other Chironomid flies not quite reproducing by paedogenesis but reaching pupal stages before giving birth, rather than reproducing as larvae as in true paedogenic cycles. This suggested to Darwin that the Coccidae could be an intermediate stage in the evolution towards a fully contracted and potentially permanently juvenile life-cycle as found in fully paedogenically reproducing mycophagous Cecidomyiidae. Darwin also suggested that the tendency towards co-existence between a contracted asexual female juvenile cycle and an expanded sexual imago life-cycle could be common in Diptera (Darwin 1859). This much does appear to be correct (Gravrilets 1997) and also highly suited to life within heterogeneity-

enhancing fungal hosts in decomposing wood (Crawley 1983, Ulrich 1943, Gabritchevsky 1929). It was Kahle and Springer who worked on the ecology and phenology of paedogenic cecids (Kahle 1908, Springer 1917). Unfortunately they lacked knowledge as to whether the cecids were mycophagous or xylophagous. The results presented in chapter 3 confirm the periods of most active paedogenic larval activity as being in early spring and again in autumn and reinforce the suggestions that water availability and temperature fluctuations play crucial direct and indirect roles in cecid larval development via conditioning of wood-rotting fungal mycelia (Springer 1915, Kahle 1908, Nicolei 1961, Varley, Gradwell & Hassell 1973). Emergence trap results in chapter 3 also confirm work by Nicolei and Ulrich of a cecid imago apring peak, thought to be induced by a series of cold nights and hot days (Ulrich 1943). It was Ulrich who found that he could influence the proportion of imago production in cultures of intermediately-sized mother larvae via varying incubation temperatures. According to Ulrich, imagoes were never produced from large and small extreme sizes of mother larvae (Ulrich 1943). The adult cecids trapped in chapter 3 were not of cecid species with paedogenic components to their life-cycles. Thus imago production for *Brittenia fraxinicola* remains rare and illusive (Wyatt 1967). Work presented here indicates that a constant incubation temperature of 25 °C maintains perpetual states of paedogenesis with Heteropezini species of *Heteropeza pygmaea* and *B. fraxinicola* as no adults or pupae were ever found. However, very occasional adults were produced at this temperature were from the species of Lestrimiinine *Mycophyla speyeri*. Since it is known that imagos are indeed also occasionally produced in the Heteropezini, I suggest that sub-families of Cecidomyiidae may differ slightly in their exact developmental cues for rare imago production. Work presented in chapters 4 and 5 reinforced previous results by Ulrich, Nicolei and Gabritchevsky that body length, life-cycle speed and offspring numbers could be altered by abiotic environmental conditions and biotic context such as fungal host mycelium (Ulrich 1943, Gabritchevsky 1929, Nicolei 1961, Wyatt 1961, 1963, 1967), though work in this thesis was the first in this regard to use an ascomycete fungus culture, and the first to use the basidiomycete *Vuilleminia commedens*. Moreover, previous work had not measured cecid life-cycle responses to interactive states of mycelia, nor to mycelial systems with PSP. Work presented here on the "jumping" dispersal mechanism of *B. fraxinicola* represents the first attempt to derive any data from this behaviour for any insect larvae as far as I know. On the basis of observations, I suggest that this behaviour is not confined to cecids but often appears in highly aggregated insect larvae with continuous migration to nearby ephemeral sink habitats (Wyatt 2000), itself taken as one line of evidence for site-dependence (Rodenhouse, Sherry & Holmes 1997, Diffendorfer 1998, Wertheim *et al.* 2000, Ranta, Kaitala & Lundberg 1998, Ranta *et al.* 2000, Lundberg & Palmqvist 1998). Though not confined to cecids, dispersal by larval "jumping" appears to be highly effective and well developed in cecids, especially mycophagous paedogenic genera (Springer 1915, Mamaev & Krivosheina 1993). I suggest that such activity in cecids and other Diptera may serve as an important means of "leapfrogging" through successive sink habitats until the next source habitat is encountered. This is especially so when considering that small larvae may be blown by the wind into which they "jump" for several hundred metres in the canopy, pointing to a combined active-passive transport system (Dajos 2000). Work presented in chapters 4 and 5 regarding cecid foraging behaviour may also help to reconcile two opposite suggestions by Kahle and Springer regarding observed movement of larvae towards or away from light. Springer suggested that cecids were attracted towards light, whereas Kahle thought that cecids were repelled by light (Springer 1915, Kahle 1908). As reported in chapters 4 and 5, differences emerge in foraging behaviour, speed, curvature of trails between young first-instar *B. fraxinicola* and mature mother larvae. I suggest that such differences extend to their phototaxicity with young larvae being positively phototactic whereas mother larvae are possibly negatively phototactic.

This would enable young larvae to emerge and "jump" from bark into passing air currents, and enable mothers to locate dark recesses within PSP zone architecture in which to become dormant resting-mothers or to give birth. In general, the relative lack of developmental determinacy and the phenotypic plasticity of cecid development appears to be well suited to life in heterogeneous, ephemeral and decomposing conditions of their indeterminate mycelial hosts (Nicolei 1961, Crawley 1983).

8.3.2.2.3 Sciaridae and Mycetophilidae

Work presented in chapter 5 reinforces observations made by Boddy, Coates and Rayner that larval foraging by Mycetophilidae and Sciaridae is enhanced and concentrated at interactive zones between mycelia of the same species where one finds mycelial somatic incompatibility zones (Boddy, Coates, Rayner 1983). The same observation was repeated on a range of intra-specific pairings for basidiomycete *Stereum hirsutum* and Ascomycetes *Phlebia rufra*, *Phlebia radicata* and *Hypoxylon serpens*. Although these observations by Boddy, Coates and Rayner applied to both Ascomycetes and Basidiomycetes, no experimental work, up to now, has looked at pairings between the two fungal sub-divisions. Mycetophilid fungus gnats of genus *Bradysia sp.* were found to synergistically interact with *Fusarium sp.* on alfalfa (Hatcher 1995), supporting evidence of synergistic interactivity between fungal and insect components of tripartite systems. Synergistic interactivity was found in work between paired Ascomycetes and Basidiomycetes, which, on producing PSP zone, appears to attract *B. fraxinicola* larvae, especially mother larvae, as presented in chapters 6 and 7.

8.3.2.2.4 Insects and mycelia

It has been suggested that the mycelial component (as opposed to fungal reproductive tissues) of insect-fungal interrelations has been largely ignored (Harper 1977). Indeed, the "utilisation by insects of small and often scattered fungal (mycelial) resources that abound in terrestrial ecosystems is largely uncharted territory" (Hammond & Laurence 1989). Furthermore, the study of bipartite reciprocity in terms of effects of insects on fungi has received little attention bar a few exceptions such as the work on ciid beetles inhabiting the basidiomycete *Coriolus versicolor* (Guevara, Rayner & Reynolds 2000). It has been recognised that the developmental biology of the fungal kingdom centres upon environmental induction of mycelial cell wall metabolism and consequent formation of collective mycelial phenomena such as chords, fans, fruiting bodies and partitions such as PSP (Wessels 1990, 1993, Rayner 1992 a, b, c). At the metabolic scale within hyphal protoplasm, such processes affecting variable hyphal insulation are thought to be governed by feedback through channelling of oxidative metabolism (Rayner 1996 b, 1997, Rayner, Ramsdale and Watkins 1995a, b, Rayner *et al.* 1999 b, c, Wilson 1992 b). Therefore any environmental interaction that alters the boundaries around mycelia, whether of collective or individual hyphae, may consequently affect oxygen stress levels, hyphal insulation and emergent mycelial developmental form. This much has been reinforced by the work presented here in interrelations in the *Brittenia-Hypoxylon*-PSP-*Vuilleminia-Corylus* system. It is also of great interest that mycetophilids of genus *Bradaysia sp.* were found to be attracted to and tunnelled through somatically incompatible reactive interfaces between intra-specific pairings of both Basidiomycetes and Ascomycetes (Boddy, Coates & Rayner 1983). To Boddy, Coates and Rayner this observation increased the likelihood of reciprocal developmental feedback of insects on fungal development with potentially wide-ranging repercussions on the development of wood-decay fungal communities (Swift & Boddy 1984, Coates & Rayner 1984). This possibility was reinforced by finding a reciprocal developmental feedback between mycelia of the beech bark basidiomycetes *Septobasidium sp.* and *Uredinella sp.* and the scale insect

Aspidotus osborni (Cooke 1977, Cooke & Rayner 1984, Rayner 1997). Here a small proportion of scale insects from tight clusters are parasitised by biotrophic fungal haustoria only once insect stylets have been inserted through bark into the living phloem of the tree. It has been suggested that the overall relationship between these scale insects and their basidiomycete mycelia is mutual since the mycelium has been shown to protect the insect clusters from predation. Such tripartite interactions also suggest that relations between taxa can be scale, contextually and temporally dependent (Hatcher 1995, Rayner 1997). Work presented in this thesis demonstrates for the first time that inter as well as intra species and inter sub-phylum interactions can be altered by insect life-cycles and foraging with respect to mycelial development of pigments, PSP zones and insulated mycelium. Also, this work demonstrates for the first time that insects can affect the accumulation of durable PSP residues within decaying wood. The finding that emergence of latent fungi from quiescent stages can overpower the effect of two month's contact with mycelial cultures on agar plates reinforces the previous suggestions of Hickin and Rayner that latent quiescent mycelia of ruderal decay species, such as *Hypoxylon fuscum*, *Vuilleminia commedens* and *Stereum sp.* colonise dead heart-wood long before the hazel pole has become dysfunctional (in terms of transpiration) and the phloem has died. The *Brittenia-Hypoxylon*-PSP-*Vuilleminia-Corylus* system represents a model for understanding an intriguing area of biology that has remained hidden from ecological investigation up to now. This model clearly provides insight into reciprocal effects of insect and fungal mycelia in rotting wood (Swift & Boddy 1984, Bruns 1984). I hope that this thesis serves to break ice and allows this important ecological theme to be researched further to shed light on decomposition, a process on which so much of the world's biodiversity depends.

8.3.2.2.5 Insects from fruiting bodies

The greater proportion of research on direct interactions between fungi and insects is in relation to the development, maintenance and stability of insect metapopulations on or emerging from, a variety of fungal fruiting bodies found naturally growing or used as baits in different habitats (Grimaldi, Jaenike 1984, Grimaldi 1985, Gilbertson 1984, Kimbrough 1984, Guevara & Dirzo 1999, Worthen, Mayrose & Wilson 1994, Worthen, Bloodworth & Hobbs 1995, Worthen Carswell & Kelly 1996, Worthen 1989 a, b, Worthen & Moore 1991, Worthen *et al.* 1995). Most insect populations in temporary and smaller sized agarics are dipteran, particularly *Drosophila sp.,* whereas beetles are important in more persistent and larger sized brackets. Work presented here supports the suggestions of Worthen, Carswell and Kelly on the importance of scale when considering insect-fungal relations, in that there is a tendency for greater numbers and species of larger insects to emerge from larger fungal units. Here I use the term "unit" loosely because work in this thesis has investigated fungal mycelia, whereas most previous work has been carried out on fungal reproductive units. As pointed out by Guevara, Rayner and Reynolds (2000), their work was amongst the first to demonstrate an effect of caprophagous insects on fungal reproduction - an effect that previous workers had suggested would not be significant (Hanski 1989). Work presented here suggests that despite differences between types of fungal unit investigated, cecids may have much in common with *Drosophila* with respect to population dynamics. I suggest that mycophagy in cecids and *Drosophila* produces similar tendencies regarding highly correlated, spatially aggregated, resource partitioned, source-sink, site-dependent distributions (Ranta *et al.* 2000, Wertheim *et al.* 2000, Shorrocks & Sevenster 1995, Rodenhouse, Sherry & Holmes 1997, Lo-Farro & Gomulkiewicz 1999, Worthen 1989 a, b). I suggest that particular parallels can be made between distribution of *B. fraxinicola* in terms of the four criteria to be satisfied for site-dependence (Rodenhouse, Sherry & Holmes 1997). These are predictable sites **(1),** different site suitability **(2),** non-

random frequency of site-occupation **(3),** and increased occupation of lower quality sites as population rises **(4).** Generally it seems that decomposing systems generate conditions where tight clumping of larvae occurs, as shown from work on flies in rotting carrion and wood (Ives 1995, Morin 1999). I suggest, therefore, that the mycelial component of habitats that these larvae share must channel development of larval populations, via natural selection, into site-dependent aggregations which mirror the heterogeneous context dependency of fungal hyphae (Davidson *et al.* 1996). Another broad agreement is between work presented here and that of Martin, Hardin and Sevenster, who suggested links between heterogeneous patchy resources, aggregative co-existence mechanisms, which reduce competition in concert with resource partitioning and allocation of refuges in terms of predation-free or toxin-free spaces (Martin 1979, Hardin 1960, Wertheim *et al.* 2000).

8.3.2.2.6 Indirect effects

Work presented here suggests that the propensity for indirect and higher order effects increases with the number of components in a system and the degree of reciprocal interactivity through which they relate. The *Brittenia-Hypoxylon*-PSP-*Vuilleminia-Corylus* system is the first pentapartite insect-fungal system to be investigated in terms of reciprocal effects as far as I am aware (Hatcher & Ayres 1997, Hatcher 1995, Morin 1999, Worthen & Moore 1991). It is thought that with increased interactivity of direct and indirect kinds, the system's phase space has an increased likelihood for non-additive and synergistic or non-linear effects (de Nooj, Biere & Linders 1992, Morin 1999, Maurer 1999). These effects may be at the metabolic scale, leading to phenomena such as induced plant resistance via increased enzyme and oxidative metabolism, with a negative effect on insect development (Bi, Felton & Mueller 1994, Hatcher 1995, Hatcher & Ayers 1997), or these effects may be manifested at the population scale, leading to emergent structures, boundary and distribution changes such as within necrotrophic and saprophytic decomposition with a tendency to enhance insect development (Hatcher 1995, Leath & Byes 1973, Shinkaji, Okabe & Amano 1988). Work in this thesis points towards a seeding of the population scale shifts from the interactive events at metabolic scales, such as when protoplasm reacts to environmental stress. I suggest that the metabolic seeding of physical events at higher scales could be referred to as the synergistic seeding of synergetic emergence (SSOSE) (see previous section in this chapter for an explanation and breakdown of pentapartite interactions). Work presented here reinforces previous work by Hatcher and Ayres on tripartite systems in terms of lack of predictability of the system from bipartite or single-species studies, and in terms of metabolic scale events with changed peroxidase and catalase activities, and changed levels of H_2O_2 (Hatcher & Ayres 1997, Hatcher 1995). Finally I suggest, on the basis of this and previous work by Hatcher that increasing interactivity between ecological niche components is mirrored by increased potential for heterogeneity, synergy, indirect and cyclic effects, which conceptually links biological with indeterministic chemical and physical processes close to turbulent states (Favre *et al.* 1998).

8.3.2.2.7 Ants and termites

Comparisons between work presented here and that on ants and termites are general and twofold. Firstly, the nature of these insect colonies is highly sexually skewed towards females, with the majority of ant workers being sterile females. A second similarity appears in colony organisation through living in tight aggregations with some division of labour. As with ants and termites, cecid males are only needed for the production of male gametes, the necessity for which may be rare and environmentally induced. Regarding colony organisation for which ants and termites are famous, cecids arrange themselves in fans and, as in the case of

B. fraxinicola, columns under bark which follow paths of least resistance with mothers in the middle and young larvae towards the outside (Springer 1915, Kahle 1908). In addition, young cecids are highly mobile, explorative, expendable, dispersal units, akin to workers, whereas mature mothers are more sessile, persistent survivors, akin to queens. Additionally, comparisons can be made to the fungal components of cecid and ant/termite foraging systems (Rayner & Franks 1987, Baker 1982, Rayner 1997). I suggest that another comparison to make in light of this work relates to the role of specific structural architectures made from mycelium and its context, in which cecid larvae and ant/termite broods feed (Corbara & Dejean 1996, Creffield 1996). These architectures share a number of features that I suggest are brought about by the developmental interactivity of larvae and mycelia. These structures are highly heterogeneous and full of cavities and sub-cavities, with a high fractal dimension and a profusion of broken symmetries which shape complex arrays of gallery spaces in highly branched and networked connection (Batra & Batra 1979, Wood & Thomas 1989, Sands 1969, Ball 1999).

8.3.2.2.8. Coleoptera and fungi

Beetle-fungal relations are amongst the most widely known and most commercially important insect-fungal groups due to the vectoring of fungal propagules of pathogenic lignin-digesting fungi by bark beetles (Hickin 1975, Webber & Gibbs 1989, Swift & Boddy 1984). Examples include the *Scolytus scolytus* vectoring of ascomycete *Ophiostoma ulmi* in mycangial pouches to form the elm-lethal beetle-gallery-fungal complex famous as "Dutch elm disease" (Dowding 1984, Webber & Brasier 1984). Other "classic" fungal-insect symbiosies are common in beetles, such as the use of acquired enzymes and gut myco-flora to digest lignin when beetles exist in wood without external mycelium (Wheeler 1984, Dowding 1984), and the cultivation of ambrosia fungi by ambrosia bark beetles (Hickin 1975, Swift & Boddy 1984, Murro 1926). The *Brittenia-Hypoxylon*-PSP-*Vuilleminia-Corylus* system presented here can be compared to a variety of general and specific beetle-fungal systems. Firstly the tripartite *Gastrophysa-Uromyces-Rumex* system of Hatcher and Ayres involved changed insect development rates, mortalities and reduced fecundity when *Rumex crispus* plants were treated with the fungus *Uromyces rumicis* and *Gastrophysa viridula* beetles, as opposed to either fungus or insect on their own (Hatcher & Ayres 1997, Hatcher *et al.* 1994, Hatcher 1995). Such changes at the population scales were mirrored by metabolic and membrane-receptor changes at smaller scales (Hatcher 1995). Work on ciid beetles by Laurence, Pavour-Smith and Guevara strengthens a suggestion I make on the basis of work presented here that distribution of ciids amongst different bracket fungi is scale and site-dependent on fungal structural allocation of spaces. Larger beetles seem to occur in brackets of species or developmental stages of species where the potential to find refuge-cavities commensurate with the body sizes of the beetles is increased, whereas smaller ciid species, such as *Octotenmus glabriculus* occur in smaller primordia or thinner brackets of *Coriolus versicolor* where the larger species cannot fit (Laurence 1973, Paviour-Smith 1960 a, b, Guevara, Rayner & Reynolds 2000). Similar non-random size allocation between small and larger sized cecids seems to reduce intra and perhaps also inter specific competition in the sub-cortical cavity matrixes and PSP zones (chapter 3). Finally, the enlargement of cavity matrixes by the effect of insect development within fruiting bodies and rotting wood noticed by Beaver is strengthened by work presented here on the influence of cecids on PSP production and wood cavitation (Beaver 1989). This points to a suggestion that insects living within fungi may often dynamically affect their niche size as they grow. If so this tendency would be mirrored by gall-formation in living tissues of plants and reinforce a view of niche as a changing field of conditions which is shaped as much by the accumulated actions of its inhabitants as by initial conditions and the environment (chapter 7).

8.3.2.2.9 Lepidoptera and fungi

A small number of "micromoth"-fungal systems (Hickin 1975, Dajos 2000) can be compared to the work presented in this thesis. These are primarily moth species whose larval stages inhabit rotting bark and fungal cankers, such as the family Oecophorinidae (Powell 1971). Rabin and Pacovsky found that altered chemical composition by fungi reduced larval growth of families Geometroidea and Noctuoidea (Rabin & Pavocsky 1985, Rawlins 1984). Also possible mutualisms exist between pathogenic fungi and moths on crop plants (Hatcher 1995). Some moths eat lichens. This aspect of mycophagy has received little attention in terms of the effects of the insects on the development of lichen thalli or lichen population studies (Dajos 2000).

8.3.2.2.10 Crop plants

Hatcher has reviewed possible synergistic effects whereby larvae of the European corn borer, in maize, develop faster when there are two fungi simultaneously infecting the plants. Similarly, the development of asparagus leaf miner larvae is faster when asparagus is infected with *Fusarium sp.* necrotrophic mycelia (Hatcher 1995).

8.3.2.2.11 Biology of interactions

The *Brittenia-Hypoxylon*-PSP-*Vuilleminia-Corylus* system points towards interactive concepts of niche assemblage in which insect and fungal development can be perceived as mutually attuned (Carrol 1995, Rayner 2000) and whose mutually effected development can be compared at different scales from distribution patterns of hyphal and larval foraging trajectories (Couzin 1999, Baker 1982, Gauthreaux 1980, Rayner & Franks 1987, Rayner 1997) and metabolic pathways affecting emergent boundary structures. With cecids, as is often the case in mycophagous fly communities, multiple factors are involved at metabolic and physical structural scales for source habitat, site-dependent aggregations to become realised from initial condition sensitivity (Rodenhouse, Sherry & Holmes 1997, Wertheim *et al.* 2000, Crowley *et al.* 1997, Szekely 2000, Tanaka, Nonaka 1991, Goodwin 2000, Taylor 1999 a, Favre *et al.* 1988). Work here reinforced several lines of thought based on previous work. These were **(1)** the **importance of spaces, cavities, rot-holes and tree holes** as refuge and niche-space (Dajos 2000, Pim & Kitching 1987) **(2)** emergent physical **architecture of decomposing woody niches** (Dennis 1997, Levine 1953, Whittaker & Levin 1975, Koch 1994), **(3) the conceptual importance of considering the developmental biology of trajectory patterns** (Brown 1999, Rayner 1997, Rayner & Franks 1987, Franks 1996, Couzin 1999, Gauthreaux 1980, Gendron & Staddon 1984, Barber 2000, Fraenkel 1961) **(4)** when comparing **parallel evolution and mutual co-evolution of plants, animals and fungi** (Pirozynski & Hawksworth 1988, Pirozynski & Malloch 1988, Crawley 1983, Rayner 1997), **decompositional successions of organism assemblages** (Frankland 1984, 1988, 1992, Swift & Boddy 1984, Hatcher 1995, Bowers 1991, Morin 1999) and their effect on **r-K strategies** (Maurer 1999, Ramsdale 1996, Watkins 1998). Finally, **(5)** the **effect of heterogeneity and its generation on indeterminacy, space-filling efficiency, symmetry-breaking, synergy and branched, networked space (structure which expands and maintains coherence – as in "Expansion under contraction" – buffering the speed of energetic and entropic increase, thus enabling biological structures to survive environmental challenges)** (Brooks & Wiley 1988, Salvador 2000, Sterelny & Griffiths

1999, Bascompte & Sole 1995, Sole & Bascompte 1998, Sparrow 1999 a, b, Moller & Swaddle 1987, Geraci 1992, Frost *et al.* 1999, Ball 1999).

8.3.2.2.12 Metabolic interactivity

Changes in chemical interactions within fungal protoplasm and insect cells, often synergistic and induced by reception of environmental signals at membrane-bound proteins (Hatcher 1995, Hildebrand *et al.* 1989), appears to influence physical boundary properties such as variable thickness and length (proliferation) (Koch 1994, Shutz *et al.* 1985, Bartnicki-Garcia 1987, Cooke & Rayner 1984). Such processes are involved in phospholipid metabolism, chitin metabolism (Neville 1975, Gilmour 1965) and, as shown in this work, emergence of PSP zone metabolism at a larger scale. Development at greater scales than protoplasmic, such as collective hyphal fruiting bodies, fans and PSP zones, appears to be seeded by underlying chemical synergies of interacting metabolic pathways at lower scales (Haken 1980, Hatcher 1995, Hatcher & Ayers 1997, Hatcher *et al.* 1994). Work by Keitt, Johnson and Bar-Or suggests that this synergy, found so often as a result of chemical combinations which react to form new products which themselves influence the rate of the reaction via feedback, increases with interactivity, heterogeneity and number of metabolic entities (Keitt & Johnson 1995, Bar-Or 2000, Baskin & Norde 2000, Ben-Jacob 1997, Ben-Jacob & Levine 1998, Bascompte & Sole 1995). Heterogeneity of metabolic pathways is thought to be greatly enhanced by the action of reactive oxygen species (ROI) (Jork, Funk & Fisher 1990) and high levels of H_2O_2 (Bi, Felton & Mueller 1994). Such manifestations of environmental stress also give rise to consequential changes to membrane phospholipids (lipid peroxidation) (Salvador 2000, Byung 1993) and the structure of protoplasmic membranes (Hilderbrand *et al.* 1989), which harbour most cellular receptor and ion-channel proteins (Alberts *et al* 1989, Kordon 1993). Change to ion-channel and receptor proteins enhances metabolic diversity (Keitt & Johnson 1995, Rayner 1997, Rayner 1999) since it can lead to increased output of secondary and inter-cellular signal chemicals (Bi *et al.* 1994, Williams 1970, Cymborowski 1992, Wigglesworth 1970, Hunt 1997), changed nutrient levels (Hatcher 1995), increased levels of heat shock proteins and associated metabolic dysfunction (Byung 1993, Salvador 2000), changes to oxalic acid to calcium oxalate ratios (Taquir 1987, Hickin 1975, Franceschi & Horner 1980, Yoshihari, Sogawa & Pathak 1980, Shimada, Akamatzu & Hattori 1994, Hatcher 1995), changes to carbohydrate and ATP metabolism (Alberts *et al.* 1989, Kordon 1993), such as ADP-ribosylation of histone and other regulatory proteins (and possibly of nucleic acids) (Kappus 1997), other epigenetic events such as DNA methylation alterations (Jablonka & Lamb 1995) and enhancement of melanin and other pigment biosynthesis (Henson, Butler & Day 1999, Felton *et al.* 1994), such as a sequiturpenoid called hydroperoxylone in ascomycete fungi of genus *Hypoxylon sp.* (Edwards *et al.* 1991). This novel green secondary compound is perhaps biosynthesised from a possible precursor found in a related genera of ascomycete *Daldinia sp.* called dihydroxyperlene using quinone metabolism and dinaphyl (Hashimoto & Asakawa 1998). Other developments that occur in cell protoplasm as a consequence of oxidative metabolism are cell senescence, ageing and death (Byung 1993, Rayner 1999). Therefore, a host of compensatory metabolic pathways have evolved to act to reduce levels of reactive oxygen intermediates and H_2O_2 (Kordon 1993). These regulate against run-away increases in cellular entropy as a consequence of oxidative metabolism (Salvador 2000), which, without tempering, would lead to apoptosis (Rayner 1997, 1999). Such damage-limiting biochemistry includes a battery of antioxidants, superoxide dismutases, catalases and peroxidase iso-enzymes responsible for reducing levels of ROI (Heinfling *et al.* 1998). As such, levels of free radicals are reduced to lower levels where they are effectively of great benefit by being accommodated and perhaps even partially harnessed or channelled towards increasing metabolic and

transcription diversity (Kordon 1993, McClintock 1983). Such a schema is particularly relevant to the *Brittenia-Hypoxylon*-PSP-*Vuilleminia-Corylus* interactive system since fungal enzymatic lignin catabolism uses inter-hyphal peroxidases (Bronner, Westphal & Dreger 1991) to de-polymerise lignin's coumaryl alcohols into phenyl propanoid monomers via a reversal of the Shikimate biosynthetic pathway (Shimazono 1951, Cooke & Rayner 1984, Rayner 1988 a), which causes free radicals and H_2O_2 to be released into the mycelium and its woody context. So, for wood-decay fungi and any organisms that live in close proximity, the above issues are likely to be even more important than for other biological systems (Rayner 1997). Not only are free radicals important in causing events that lead to hyphal insulation, pigmentation and emergence of PSP, but also H_2O_2 and peroxidase are necessary as components in the sclerotization of insect chitin-protein cuticles. Evidence from work presented here from the H_2O_2 traces made by cecids through mycelia in which they forage, to the changed levels of peroxidase, antioxidants and catalase enzymes, supports the above schema and, for the first time, demonstrates the way in which PSP zone metabolism may operate in fungal systems, towards increasingly hydrophobic oxidant-sequestering pigments, creating a toxin-free refuge as well as an enemy-free space (Lawton & Strong 1981).

8.3.2.2.13 Hymenoptera and fungi

Comparisons between the cecid-fungal systems of this and previous work, and that of siricid wasps of families Sircidae and Xiphydridae, centres on the larval component of the wasp life-cycle and how this relates to its combined fungal food and home. Both cecid and Siricid larvae depend obligatorily on fungal diets, and live within decomposing wood (Hickin 1975, Swift & Boddy 1984, Dowding 1984, Cooke & Rayner 1984). The vectoring of spores or propagules of fungal mycelia from feeding sites in wood differs in mechanism but may be shared in principle. Scanning electron images presented in chapter 3 showed many fungal spores and hyphal fragments that could be seen attached to the cuticular creeping welts of *B. fraxinicola*. The oidia-carrying mycetangia of siricid larvae and adults are essentially invaginations of similar cuticular morphology as the cecid creeping welts. This suggests that facultative mutual or casual vectoring of an insect larva's microbial context may be common, and that the evolution towards specific obligate mutual relations stems from this.

8.3.2.2.14 Mites and fungi, Collembola + other invertebrates

Arachnid mites are some of the most common mycelial-dwelling mycetophagous organisms together with insects of orders Diptera and Collembola (O'Conner 1984, Athias-Binche 1989), annelid earth worms, nematode worms and many other microbes. Mites have been shown to possess some parallels with the system presented here. There is good scope for research regarding reciprocal developmental feedback between mycelia and smaller sized mycelial inhabitants (Swift & Boddy 1984, Cooke & Rayner 1984). Mites range from obligate mycetobionts, for example *Siteroptes sp.* which does not develop on its host plant cotton, without the plant being infected by mycelium of *Nigrospora sp.* (Hatcher 1995) and may act as a vector of spores. Other mites are less mutually or commensally symbiotic with their fungal interactants, these being facultative mycetophiles, and accidental mycetoxenes (Athias-Binche 1989). Work presented here on the *Brittenia-Hypoxylon*-PSP-*Vuilleminia-Corylus* system compares well to that of mycetophagous orbatid mites that inhabit darkly pigmented cavity systems in decaying wood. Like cecids, development of mites is also rich in environmental cues. Mites appear to share temperature as a major developmental cue with mycetophagous cecids and *Drosophila* (Ydergaard, Enkegaard & Brodsgaard 1997). Like nematodes with

respect to female-biased sexual skews in highly aggregated populations, mycetophagous mites also share the same gender skews with mycetophagous Diptera and also aphids, with whom paedogenic cecids share female-based asexual reproduction with developmental cues from the environment which determine when to produce sexual alates (Lussenshop 1992, Margan & Bruman 1994). Another parallel between the cecid-fungal and aphid-fungal relationships, as reviewed by Hatcher, is exemplified by a tripartite interaction with *Aphis fabae* which increases its population development on leaves through an indirect facultative mutualism with the fungus *Uromyces sp.* (Hatcher 1995, Chang & Thrower 1981). Overall, such comparisons suggest that small organisms inhabiting heterogeneous niches, such as forest mycelia or spatially clumped annual plants (as with parthenogenic aphids, Cognetti 1965), share a plasticity of genome, phenotype and a tendency towards asexual reproduction which is likely to be co-evolved to suit the very heterogeneous and patchy conditions which their fungal and plant hosts provide (Moller & Swaddle 1997, Crawley 1983). Moreover, convergence of developmental strategies strengthens speculation of mirrored tendencies in developmental indeterminism between co-existent, symbiotic taxa (Tokeshi 1999).

8.3.2.2.15 Wood decomposition

If we were to weigh out all the molecules in living systems, it is thought that lignin would probably be the most abundant bio-synthesied molecule on the terrestrial surface of the planet except water (Rayner 1988, Rayner 1998 b, Rayner & Way 1999). It is a major reservoir of fixed carbon and continued cycling of lignin's bio-synthesis and decompositional ecology is of major significance to all of the biodiversity that lives on planet Earth, particularly to insects and fungi which live inside woody plants (Wilson 1992 a, Hawksworth 1990, 1991, 1998, Rayner & Todd 1979, Rayner 1988, 1993, a, b). Work presented here and previous workers show dead wood and bark not only as a food source on which and through which insects burrow, but as a dynamic context which accumulates changes from the passage of living ecological reactions within it (Dajos 2000, Brooks & Wiley 1986, Swift & Boddy 1984, Rayner 1988). Dead xylem becomes perceived therefore as an ecological catalyst of phenomenal surface area for microbes and insects to live inside, especially for microbial communities that can ramify their collectives throughout the pits and fissures of xylem's hollow reticulate-architecture. The process of releasing water and oxygen from catalase reactions on H_2O_2 at inter-specific mycelial interfaces, and the accumulation of PSP zone hydrophobins and crystals, perhaps of oxalate (Traquair 1987), suggests a mechanism of physical forces seeded from interactive metabolism, emerging from combined chemical initial conditions with abiotic fluctuations.

This coupling of environmental fluctuations with varied chemical initial conditions initiates differential and varied expansion, contraction and cracking of wood to form initial cavitation and associated loose bark. Into such an array of interconnected small cavities can fit and forage the primary ruderal colonists of insect fauna decompositional successions that will accompany successive waves of fungal floral interactants until most of what is left is accumulated PSP zone structures, and what is not PSP has been recycled into soil. I suggest that amongst the first insects to gain entry into the sub-cortical cavity matrix are first-instar paedogenic cecid larvae (chapter 3 and 7). Larval development increases PSP zone formation that appears to enhance further cavitation around the growing and otherwise trapped cecid larvae (chapter 7). This suggests that the cavity-size may increase with the growth of cecid larvae (chapter 3). In this way, seeing parallels with utilisation of living plant metabolic pathways in order to produce living plant cavities (galls), the fly family Cecidomyiidae could really be said to be specialised "**cavity makers**", though in truth I suggest that

they only influence and perturb the metabolism of their hosts in order to enhance the production of cavity spaces in which they can develop.

When one considers these possibilities, it seems that paedogenesis in cecid reproduction can be explained as a brilliant evolutionary development for first wave insect colonists of the mycelia of ruderal primary fungal colonists, particularly within Ascomycetes like *H. fuscum* which are most chemically versatile at sequestering free radicals and building PSP. Despite these succession dynamics, fungal mycelia are brought into an intimate association with mycelial co-inhabitants for comparatively longer periods of time than in more ephemeral agaric fruiting bodies in which most research on effects of fungi on mycetophagous dipteran communities has been based (Dajos 2000, Jaenike & James 1991, Shorrocks & Bingly 1994, Worthen, Jones & Jetton 1998, Sevenster 1996). **Despite this difference, mycophagous paedogenic cecids share many parallels with other insects that live in dead wood. These comparisons fall into three overlapping themes.**

Firstly the increased potential for mutual ectosymbiosis between longer-lived partners in heterogeneous wood-decay relations has perhaps enhanced capacity for co-evolution (Tokeshi 1999) achieving the classic insect-fungal relationships such as ambrosia fungal-ambrosia bark beetle complexes (Hickin 1975), with the acquiring of fungal enzymes in the guts of most Coleoptera which inhabit wood (Hickin 1975, Swift & Boddy 1994, Dowding 1984), and amongst the anobid beetles, developmental reciprocity, similar to that of work presented here, of the death watch beetle *Xestobium rufovillosum* (Hickin 1975). Work by Fisher suggested that this polyphagous larva eats mycelium inside large pieces of structural timber and mature Salix, Fraxinus, Quercus and other trees (Fisher & Parkin 1930). Fisher found that the development time for larvae ranged from 10 to 55 months, and correlated development time with structural integrity of the wood. The more rotted the wood, and the more likely it is to have a developed fungal community, the quicker the development speed of death watch beetle larvae (Fisher 1940, 1941). Work by Becker suggested similar relations regarding the development of long horn beetles with mycelium (Becker 1938). Campbell and Bryant confirmed much of what Fisher had proposed for the death watch beetle, although they remained unconvinced about the mycelium as being the primary food source for larvae (Campbell & Bryant 1940).

Particularly relevant to cecid life-cycles is the observation by Fisher that the adult death watch beetle seldom flies, relying mostly on walking to colonise new local sites. This bears some parallel to the reduction of life-cycle by paedogenic cecids with very infrequent imago production, and the female sexual skews so often observed with mycophagous dipteran populations. It has been suggested that the evolution towards life-cycle reduction is linked to asexual reproductive strategies and dispersal requirements (Gavrilets 1997, Morin 1999, Holt 1994, Odum & Biever 1984, Atkinson & Shorrocks 1984). Where distribution of food resource is ephemeral, patchy and heterogeneous, as exemplified by fungal mycelia, I suggest that conditions are satisfied to select for a tendency towards female-skewed populations (Starzyk & Witowski 1986) with rare needs of long distance dispersal, with a tendency towards asexual reproduction and a shortening of the life-cycle by reducing development of number of moults and metamorphic stages. This thereby speeds up generation time so as to give rise to populations of very high potential growth rates once they encounter the multiple conditions which satisfy their source habitat requirements (Worthen, Bloodworth, Hobbs 1995, Wertheim *et al.* 2000). Evidence from cecids which supports this view is that the life-cycles of female paedogenic mycophagous genera all have the fastest generation times and the fewest number of moults and

only produce imagos under exceptional circumstances, which, when formed, are very rarely male. *Heteropeza pygmaea* has no moults at all whilst *B. fraxinicola* has two (Nicolei 1960, Wyatt 1967).

The **second** theme of interest regards non-mycophagous cecids, i.e. those which make galls in living parts of plants. Cecids have been shown to influence the auxin hormonal balance around three developing tissue layers. Occasionally these cecids are found with fungi developing with them inside the living plant galls (Gould & Steiner 1984, Batra & Liechtwardt 1963). These fungi do not ramify through the gall unless the insect dies or until the insect has left the gall. Perhaps some cecids are cultivating plant biotrophic fungi within their galls and harnessing the chemical versatility of the fungi to interfere with the plant metabolism. Experimental work in this regard needs to be carried out.

Thirdly, two very special habitats appear in dead wood, whose importance is promoted by work presented here. These habitats are tree holes and bark (Dajos 2000, Moffet 1993). The development of tree holes mirrors that of the developing bark cavities. Both are a function of time, scale and r-K tree succession (Dajos 2000). Many insects live in bark, either on the reticulate crevices on its surface (Wilson 1992 a, Dajos 2000) or in multiple layers underneath (Irmler, Heller & Warning 1996). It has been demonstrated that there are four insect faunal successions involved in bark decomposition. Firstly Sciaridae, secondly Mycetophilids, thirdly Sciarids and Mycetophilids, fourthly soil dwelling invertebrates including Annelid earthworms (Irmler, Heller & Warning 1996). I suggest that perhaps no mention of cecids appears here because, as in chapter 3, the data are based on emergence of adults. Similarly, investigations of tree hole invertebrates do not mention cecids (Dajos 2000, Vaillant 1978). I suggest that this is so due to the focus of tree cavity ecology on large scaled tree holes and tree boles (water-filled recesses at the junction between main and secondary trunks) which house much of the climactic biodiversity of many birds, tree-frogs and even small primates whose lives depend on the refuges provided by large cavities in wood (Dajos 2000, Moffett 1993). However, work presented here demonstrates that at much smaller scales, tree holes and "rot-holes" (Dajos 2000) are analogous to cavities inside the wood which house much invertebrate life and which are seeded by wood-decay fungal communities. Supportive evidence of the seeding of tree hole potential from initial fungal activity comes from studies in Canadian boreal forest which shows that the accommodation of a keystone conservation species, the flying squirrel *Glaucomys sp.* in large cavities in dead standing softwood trees or snags, depends on avies-mites which evict the Greater Pickerell woodpecker from cavity-nests. These cavity-nests are excavated from heartwood fungal decay columns in mature softwood trees, without which, it has been suggested, the wood would be too hard to excavate into a nest-cavity. Pickerell woodpeckers of the Atlantic maritime forest have even been observed tapping at the base of young spruce trees long before they would be of use for cavity sites. This behaviour has lead to a suggestion that these woodpeckers thereby inoculate the heart-rot fungus of their future nest-cavities in trees up to several generations in advance of the tree maturity required for cavity-nest excavation (Taylor 1993). I suggest that similar events occur at smaller scales, particularly with mycophagous insects. It has been estimated that 25 per cent of the global beetle diversity lives in rotting wood (Blaney 1999, Dajos 2000, Nilson, Hedin & Niklasson 2001, Ehnstrom 2001, Blackman & Stage 1924, Blackwell 1966, Berryman 1989). This, and similar stories of fungal initial conditions seeding the potential for future ecological events (Tosheki 1999, Torgersen & Bull 1995, Torres 1994), provides a conceptual frame around this thesis on the importance of interactivity and space-making cavitation processes in ecological systems.

8.4 Implications – thoughts of interest to contemporary debates

This study adds new perspectives and considerations to the type of change which relationships induce on interactants via the emergence of synergetic structure from zones of reciprocal perturbation. The process which gives rise to these patterns appears to involve a change in scale from chemical metabolic to emergent physical properties of polymerised macromolecules, such as those found in the PSP zone. This has been termed **SSOSE** (this chapter section 8.1) and reinforces previous work by Crawford 1989, 1992, Godbillion 1983, Colwell & Lees 2000, Haken 1980, Gavrilets 1997, Frost, Hawkins, Johnson & Burrows 1999, Marshall 1980, Favre *et al.* 1988, Keitt & Johnson 1995, Lewin & Regime 1998, Lewis 1987, Lucking 1998, and McDonnell-Alexander 1999. Another way to think about this is that (living) interactive entities in states of symbiotic synergy drive diverse dimerisation (interactive coupling) towards an increased emergent diversity of the system's surfaces (topological stretching and foldings). Since the primary rules governing such patterning systems are thought to be universally thermodynamic and entropic (Brooks & Wiley 1986, Thornton 1995, Strogatz 1994, Thom 1972, Anderson & Bunas 1993, Acheson 1990, Prigogine 1980, Ball 1999, Bagnold 1941, Tritton 1977, Bonsall, Jones & Perry 1998, Baskin & Norde 2000, Berry 1998, Bertalanffy 1968, Bhatia & Szego 1970) and it is the durability of coherent forms under perturbation from the environment that are selected, then it is surely our challenge to incorporate existing knowledge on how nucleotide metabolic informatics plays a role in enhancing (living) system durability and diversity of solutions to environmental problems with an inherited molecular language regulatory feedback process (Regev, Lamb & Jablonka 1998, Jablonka & Szathmary 1995, Alexandrov 1977, Andrews 1995, Arking *et al.* 1996, Aronovitch *et al.* 1984, Arechi 1996, Berteaud & Alfsen 1976, Cohen & Honslay 1985). As such, mycophagous cecids could provide an excellent model system with which to bring together a synthesis between evolutionary theory and the diciplines of genetics, ecology, symbiosis, phenotypic, behavioural and developmental biology.

The intimate proximity of fungal hyphae to plants and other organisms within wood and soil has endowed fungi with their propensity to form many types of co-existent and symbiotic relationships with other taxa (Sapp 1994, Smith 1987, Tokeshi 1999). Such inter-taxonomic relationships are most notable with plants through the evolution of mycorrhizae from which an estimated 75 per cent of terrestrial plant species gain enhanced phosphate and nitrogen nutrition (Cooke & Rayner 1984, Rayner 1995, Hawksworth 1991). The cultivation of fungus gardens of leaf cutter ants *Atta sp.* have been well studied (for example Huxley 1949, Maynard-Smith 1997, Cooke & Rayner 1984, Pirozynski 1993). Indeed, it has been suggested by mycologists and lichenologists, and those ecologists who study tropical canopy biology, on considering the amount of geological time in which fungi, plants and insect taxa have co-existed (Stubblefield *et al.* 1987, Moffett 1993), that the extent of commensal and mutualistic symbiotic relationships has been greatly under-estimated in the biological sciences (Tokeshi 1999, Smith 1981, Poelt 1990). This idea seems to represent a philosophical view that is perhaps as old as the notion of evolution itself. Even Humbolt (as reported by Botting 1973) and Wallace (as reported by Harley & Smith 1983 and George 1964) advocated such liberatory views, as exemplified even in Darwin's writings:

"How fleeting are the wishes and efforts of man! How short his time! and consequently how poor will be his results, compared with those accumulated by

nature during whole geological periods!.....our profound ignorance on the mutual relations of the inhabitants of the world at the present time, and still more so during the past ages....Slow though the process of selection may be, if feeble man can do much by artificial selection, I can see no limit to the amount of change to the beauty and complexity of the co-adaptations between all organic beings, one with another and with their physical conditions of life, which may have been affected in the long course of time through nature's power of selection, that is by the survival of the fittest....Let it also be borne in mind how infinitely complex and close-fitting are the mutual relations of all organic beings to each other and to their physical conditions of life....and consequently what infinitely varied diversities of structures might be of use to each being under changing conditions of life....We have good reason to believe....that changes in the conditions of life give a tendency to increased variability....by conditions changing, variations would be made newly available to natural selection" (**Darwin 1859**).

Modern-day ecological investigations suggest several lines of evidence that through inter-taxonomic intimacy over the course of evolutionary time, many differing co-evolutionary and symbiotic relationships have been established by fungi (Smith 1981, Smith & Douglas 1987, Fager 1964, Astatt 1988, Pirozynski 1988). These affect dynamic qualities of niches in which associants dwell (Guevara 1998, Rayner 1998 a, Ahmadjain & Paracer 1986, Ainsworth *et al.* 1990, Anderson & Ineson 1984, Ingham 1992, Klironomos & Kendrick 1995). Fungi have a profound effect on the niches in which they dwell over time (Dornieden, Gorbushina, Krumbein 1998). This has led to a suggestion that the entire concept of at least "fungal niche" could be viewed as dynamic (in terms of classical dynamics – see Thornton 1995) (Guevara, Rayner, Reynolds 1998, Couzin 1999, Franks 1996, Pirozynski, Hawksworth 1988, Pirozynski, Malloch 1988). This points to a concept of niche as composed of interactive and changing media (Pippard 1985, Thom 1972, Favre *et al.* 1988). I suggest that niche be seen as an expansive, foldable conduit of abiotic and biotic conditions within which symbiotic organisms are incumbent to varying degrees, and develop their distribution patterns under fluctuating regimes which subject the phenotype to environmental challenge (Ball 1999, Thom 1972, McClintock 1983).

Co-evolution has been mooted as a strong factor in the interactive biology of plants and fungi, and between plants and insects (Pirozynski & Hawksworth 1988). However, co-evolution has not been well demonstrated between insects and fungi, a field that would surely benefit from further research. The co-development (development in parallel) of hyphal wall and larval cuticle is most likely in situations when, as in this work, insects and wood-decay fungi co-exist. The reactive mycelium effectively encloses incumbent and chemically-sensitive larvae. Any chemical change caused by the larvae is bound to have some effect on the surrounding mycelium and *vice versa*. Furthermore, it has been suggested that over half the global insect species co-exist with fungi for at least part of their life-cycle (Pirozynski & Hawksworth 1988). The majority of insects inhabit niches that are at least partially decomposing for a significant phase of their lifecycle. Therefore larvae from rotting wood and leaf litter not only enable good estimates of insect abundance and

diversity within a forest ecosystem (Wilson 1992 a, Roper 1999, Pirozynski & Hawksworth 1988), but also live in close contact with fungal enzymes, proteins and fungal secondary metabolites that may affect their development, growth and behaviour. Comparisons between insect and fungal development can also be made metabolically when considering insect exoskeleton chitin and constituents of fungal chitin cell walls. This may parallel the simultaneous utilisation of Shikimate lignin metabolic pathways (Anderson & Beardall 1991, Rayner 1988) between wood-decay fungi and plants. The chitin-producing enzymes (Stirling, Cook & Pope 1979) that fungi and insects secrete may be similar in structure and function. Chitin-producing enzymes are found on fungal and insect membranes and outer surfaces (Roitberg & Isman 1992, Neville 1975). Fungi and insects share a simultaneous need of chitin-producing enzymes as well as peroxidases and catalase systems through which H_2O_2 is channelled into polymerising or de-polymerising reactions (Porter & Jaworski 1965, Asmus 1992).

The involvement of insects and fungi in interactive secondary metabolic pathways is perhaps an intriguing possibility (Wigglesworth 1970). The redox biochemistry of lignin and cellulose catabolism produces H_2O_2 (Geisebach 1981, Grierson & Covey 1988, Godfrey, Muller & Kraaijeveld 1999, Erikson 1998, Reid 1995). This is a potent producer of free radicals and ROI if left unameliorated (Kirk & Fenn 1982). At this biochemical scale, H_2O_2 will not distinguish between insect and fungi. Like water, H_2O_2 can negotiate most semipermeable membranes. H_2O_2 may be one metabolite which is small and diffusable enough to act on both taxa by increasing insulative defence via increased free radical mediated cross-linking of chitin residues in insect cuticles and hyphal walls (Mswaka & Magan 1998, Wilson 1992 b, Yu 1993, Thompson, Legge & Barber 1987). I suggest that it could be that several redox reagents and hormones produced in fungi and co-existing insects act on their metabolic target sites irrespective of which taxa they were produced in. Work strengthening such suggestions shows how fungal metabolites can act as non-specific gene regulators (Stadler *et al.* 1988 a). This is relevant to work presented here on the basis that Ascomycotina Xylariaceae, of which *Hypoxylon* is a genus, produce a huge diversity of secondary metabolites with some structural homology to steroids (Whalley & Edwards 1987, 1995). Cecids are peculiarly resistant to toxic effects of secondary compounds and insecticides (compared with other insects) (White 1977). At the same time a large variety of Diptera are thought to be associated with Ascomycetes (Kimbrough 1984) (whereas proportionately more beetles are thought to be associated with Basidiomycetes). Reciprocal metabolic feedback across membranes and walls may be one possible mechanism that allows fungal and insect development to cause reciprocal effects in parallel to what we know about the developmental feedback between plants and insects (Williams 1970, Went 1970).

This project applied a systemic way of thinking about the dynamic relationship between an organism and its abiotic and biotic context. Here it is necessary to distinguish context, which incorporates interactive abiotic with living systems, from external environment, which is often thought merely to impose conditions on an organism's development (but which I suspect is also interactive from a greater spatio-temporal scale than an organism's context). My approach to the investigation was to get to know my study site intimately, by spending much time there in the initial phase of research, monitoring contextual change that provided ranges of scales, insights and perspectives with which to proceed to study populations of co-habiting insects and fungi. The suggestion I put forward in chapter 1 was that Basidiomycotina have an enhanced hyphal boundary dexterity when compared with Ascomycotina (Rayner 1997, Shutz *et al.* 1985, Andrews 1995).

Hyphal boundary dexterity is the ability to increase or reduce hyphal wall-matrix leakiness in response to oxidative stress (chapter 1, figure 1.2) (Jelinsky & Samon 1999, Rayner 1994, Yu 1993). Oxidative stress, manifested through reactive oxygen intermediates (ROI) and free radicals within protoplasm or peroxisomes and mitochondria, punctures membranes and disrupts biochemical pathways and leads to cell senescence and death through apoptosis (Thrane 1998). Basidiomycete hyphal boundary dexterity has also led to the emergence of mycelial structures such as rhizomorphs, chords and large bracket or toadstool-shaped fruiting bodies as secondary effects (Watkinson 1979, Wessels, Mol, Sietsma & Vermenler 1990). It has been implied by Rayner, Watkins, Crowe and Ramsdale that Basidiomycetes use their hyphal boundary dexterity as a first defence against stress before resorting to latent chemical versatility. Chemical versatility means the defence of oxidatively stressed protoplasm with inter- and intra-hyphal chemicals and enzymes such as antioxidants and superoxidases. Basidiomycetes thus possess both potentials of stress alleviation to negotiate local environmental heterogeneity (Rayner, Ramsdale & Watkins 1995, Rayner, Grifith & Ainsworth 1995, Rayner, Beeching & Watkins 1995, Davidson *et al.* 1996, Crowe 1998).

On the other hand the Ascomycetes generally lack the degree of hyphal boundary dexterity that Basidiomycetes possess (Hawksworth 1987, Kimbrough 1984). Instead, as the suggestion goes (chapter 1, figure 1.2), they rely on induction of highly developed chemical versatility to adapt to oxidative stresses (Rayner 1994 b). So Ascomycetes use chemical versatility (Hashimoto & Asakawa 1998, Lichter & Mills 1998) as a first defence since their hyphal boundary dexterity is somewhat limited. This biochemical distinction between the two fungal sub-divisions (Demain 1996) also explains why a large proportion of Ascomycetes are deeply pigmented chemically (Sharland 1987, Stadler *et al.*1998, Rayner 1994 b), for example with melanins, flavenoids and coumarins (Henson, Butler & Day 1999). Many redox chemicals (for example Gk-Beta-carotene and chlorophyll) are coloured due to their partially filled electron orbitals enabling them to absorb light energy spectra. It is in the process of defending hyphae from oxidation (Wickow 1988, Walley & Edwards 1987, 1995) that antioxidant O_2^- compound structures become oxidised, revealing their colours (Ebbing 1987) by absorption of specific light wavelengths. The suggestion that Ascomycetes resort to chemical versatility also explains why they are found more predictably in damp habitats than Basidiomycetes (Rayner 1986, Correll & Gordon 1999, Lodge & Catrell 1995). This is because water's lone pair of electrons makes it a good electron-acceptor and reducing agent with some, albeit limited antioxidant properties (Ebbing 1987, Gates 1967). Water enables protoplasm to escape desiccation and consequent oxidative stress (Rayner & Way 1999, Dick 1965). Moreover, Ascomycetes lose water more rapidly than Basidiomycetes since they possess only a limited ability to prevent desiccation by re-insulating their boundaries, (Rayner *et al.* 1999, Crowe 1997). Desiccation is a fate that Basidiomycetes may avoid through hyphal boundary dexterity. This avoidance of water-stress enables Basidiomycetes to live further from water than Ascomyetes, and to be more active in time of drought. The suggestion by Rayner *et al.* also explains how Ascomycete chemical versatility may be easier to demonstrate than that of Basidiomycetes (Rayner *et al.* 1999). However, basidiomycete fungi do possess a latent chemical versatility that is only exhibited during severe oxidative stress (Rayner 1996 b) such as in interaction with another fungus (Rayner 1979, 1994 a) or whilst being lysed or ingested by animals - especially insects (Pirozynski & Malloch 1998).

A cyclic process, in terms of metabolic sensitivity to changes in tissue fluid conditions, due to the effects on inter-cellular contexts induced by cell growth coupled with folding and partitioning describes the developmental feedback cycle (Thom 1992, Jelinsky & Samon 1999, Jones & Taylor 1995, Joshi &

Thompson 1996, Labno, Morley & Tillotson 1987a, b, c, d, e). Adaptations are thought to have evolved in the form of selected population dynamics of membrane-bound proteins (post-translational modification) (Sharon & Lis 1993, Wessels 1993, 1998), or produced by altered chemical equilibria from multiply and differentially expressed proteins from RNA transcribed through changed conditions within the nucleus (post transcriptional modification). As conditions and selection pressure change, thus changing ancestrally experienced signals, a re-assortment of expressed inherited information is induced (McClintock 1983). This kind of epigenetic re-assorting occurs especially during meiotic divisions and partially in polyploidous mitotic divisions, albeit to a lesser extent, and appears to occur more in bacteria, plants and fungi (Rainey 1993, McClintock 1983,Watkins 1999) than animals. Exceptions to this lack of sensitivity on the part of animal epigenetics are an important part of this thesis. It is apparent that this kind of epigenetic shifting is what chemically (therefore environmentally) induces cellular specialisation in organism development (Slack 1998). So it is likely that organism development involves context-induced gene regulation to achieve an appropriate expression that is attuned for duration in the prevailing environmental conditions, along ancestral lines, thus increasing the chances of survival of the organism. Such developmental feedback implies that final and to some extent plastic phenotypes emerge from combinations of many information inputs ranging from past environment, as recorded in DNA and RNA (Rainey 1993, Rainey 1999), i.e. how the cytoplasm ancestrally tended to react to particular stimuli in sensual or nutritional signalling (Rose 1997, Rainey 2000). This information is expressed or presented in coupling with the stimuli (present environment), i.e. chemical or physical perturbations to the existing membranes (Thom 1992, Baskin & Norde 2000) around and within continually adjusting membranes and the cytoskeleton. Several schools of thought have it that these two sources (present and past) of contextual information thus converge on a non-linear (Jennings & Rayner 1988) self-organisation processes (Kauffman 1993). The result is self-organisation and emergence of diverse forms through thermodynamic feedback (Thom 1992, Massey *et al.* 1999, Rayner 1997).

It is therefore thought that the phenotype of an organism is an emergent state that is seeded from the interactive properties of a crucial non-linear self-organising function coupled to a series of interactive information cascades from at least three sources. These are the outer cellular fluid, metabolic-epigenetic state and gene-transcription (Holliday 1989, Ho 1993, Kaufman 1993, Rayner, Ramsdale & Watkins 1995, Rayner 1996 b ,c ,d, Rayner 1997, Rayner 1998 a, b). What is philosophically important about this school of thought is the recognition that key factors involved in development are self-organisation and emergence (Thom 1992, Kaufman 1993), which are differentially affected by the above three information inputs about the environment - one of which is provided by information stored in the molecule DNA (Rainey 2000, Rose 1997).

Developmental theory (Schwemler 1989, Tompson 1917) recalls the histories of evolution and adaptation of living and thereby evolving systems as sometimes being analogous to developmental stages through which they shift as they grow (Sussman 1964). It has been suggested that a new point to add is as follows. Underlying the process of development is another chemo-physico process of thermodynamic trans-membrane feedback (Goodwin 2000, Ho 1993) which gives rise to non-linear, asymmetric sub-cellular developmental branchings, stretchings and foldings of membranes with interactive bound proteins (Grzywana & Siwy 1997, Griffiths 1998, Thom 1973, 1992, Frelich, Sugita, Reich, Davis & Friedman 1998). Such feedback enables patterns to develop via communication between cells as they form different populations of membrane-bound ion-channels, pores, gates, glucocorticoid receptors, reticulate membrane

enfoldings, membrane motifs and other epitope ligands. These changed structures interlock with neighbouring cells and maximise potential for inter-cellular signal interactivity (Baskin & Norde 2000, Kordon 1993, Hopkins 1978) and specialisation, which involves regulation of genes as one of many interactive metabolic components.

Apoptosis, a process involving enzymatic and chemical feedback, is known to be linked to oxidative stress and metabolic dysfunction in most organisms (Rayner *et al.* 1999 b, Schwemler 1989, Handel *et al.* 2000, Schwartz 1991, Rayner 1999, Thompson, Legge & Barber 1987). To become a large assemblage of co-operating metazoan cells, the regulation of growth promotion, stasis and apoptosis become crucial in development to enable the huge changes in scale which are often necessary. This link to size of an organism is suggested in the work of Bonner who saw scale-shifts as fundamental in explaining the behaviour of cellular slime moulds over time (Bonner 1965). Associated with the emergence of non-linear patterns (Lotka 1956, Lorenz 1985, Meinhardt 1982) is the regulation of different sets of cells' inherited genetic codes, which contribute to the emergence of developmental patterns faithful to ancestral lines (Rainey 2000, Dickson 2000). Underlying the dynamic developmental pattern is an equally dynamic feedback process rooted in thermodynamic laws of physics and chemistry (Baskin & Norde 2000). Chemical communication signals may travel across and set up reaction diffusion gradients between cells and between an intricate array of multiply enfolded lipid bi-layers within cells (Kordon 1993, Haken 1980, Monroy & Moscova 1979, Slack 1998, Thom 1972, Thompson 1917, Kordon 1993, Bar-Or 2000, Rayner 1997, Kordon 1993). Seen in this way, the history of oxidative stress, feedback and topological development become hard to disentangle (Thom 1972, 1992, Rayner 1997, 1998 a, 1999). This is not surprising since both are linked by consecutive cycles of cause and effect. Ultimately, almost universally associated with change is effect, which can evolve into signal, interaction, communication, relationship and (molecular) language (Kordon 1993, McClintock 1983).

However, it is arguably by focussing attention on the secondary *effects* of developmental feedback, for example the specific sequence of DNA bases contained in a transcribed sequence, rather than looking at topologically *causal* dynamics in signals, metabolic cascades, levels of kinases, phosphatases, cyclic AMP levels and electrical polarities of cells in response to environmental concentrations of ions across membranes (Thom 1992, Goodwin 1994 b, 1998, Baskin & Norde 2000, Bartoli, Bartoli 1984, Kleijn & Leeuwem 2000, Ruthen 1993, Ho 1993, 1995 a, b) that we currently culturally perceive fates of cells as controlled from metaphorical genetic "blueprints" (Lewontin 1993) and DNA "command centres" (Sterelny & Griffiths 1999). This leads to an assumption that cellular destinies for all species have been genetically pre-determined, as implied unintentionally by Watson & Crick 1953, Wolpert 1991, Browder 1984, and Hurst 1995. Whist genes are undoubtedly key information holders in the overall chemical inventory of cellular metabolism, merely showing that by removing their function a metabolic step does not occur, does not demonstrate discrete causality of control over phenomena (Ho 1993, Rose 1995, 1997). The view of a DNA information command centre in living organisms (Sterelny & Griffiths 1999) philosophically opposes the view presented earlier where, if anything is central to the process, it is the emergent state of non-linear self-organising thermodynamic chemicals (Ho 1993, Kauffman 1993). These metabolites act synergistically and provide a sensitivity to the environment at the system periphery, thereby perturbing, channelling and influencing a host of pluralistic metabolic possibilities rather than commanding or controlling one particular step (Moller, Swaddle 1997). That the concept of "DNA-command" is misguided has been appreciated by

many in the molecular field (Lewontin 1993, 1994, Rose 1997, Ho 1993, Rose 1982, Rainey 2000, Holdredge 1996, Holliday 1989, Moxon & Wills 1999, Steele 1979, McClintock 1961). However, returning to the theme, perhaps what is actually being appreciated by those who increasingly challenge the "Doctrine of DNA" is that self-organising thermodynamic feedback processes underlie development. It is these chemical, physical and mathematical processes, not genetic consequences *per se*, which produce *a priori* physical topological shifts in protein motifs, biological membrane structures and cellular shapes. It is thought that these shifts alter the pattern of cell layerings, partitions and environmental chemical signal gradients in a developing metazoan (Rose 1997, Slack 1992, 1998), which in turn influence the regulatory pathways of genes through which reciprocal adjustments are made to membrane-bound protein populations (McDonald & Bale Watters 1998, Massey *et al.*1999, Rose, Lewontin & Kaimin 1984, Lama & Avital 1998, Rossiter 1996, Lander *et al.* 1996, Wilson & Agnew 1992).

So, this investigation has scattered some light on a range of issues in the biological sciences. However what unites these seemingly disconnected strands of thought are important ideas about the role of the process of feedback as a mechanism for reciprocal interactive relations in development of population and community patterns at different ecological scales (Whitehead 1978). **One of the major emergent themes of this study has been how a combination of signals interacts to form unique and synergetic causal networks of effects on an organism's relation to oxidative stress and how to ameliorate damage without losing the ability to forage, digest and assimilate oxidative energy.** Some think that strategies of oxidative stress amelioration, via the process of feedback, have perhaps resulted in a channelling of thermodynamic energy into growth and development over the course of evolutionary history (Thom 1992, Ball 2000, Baskin & Norde 2000, Strogatz 1994). This could lead to phylogenetic diversification and natural selection as two sides of the same coin that relates diversification to the synergetic serendipity of interactive response to surroundings and natural selection pressure induced by conditions on the former responses. Thus the most durable relationships between environment and organism-interactivity are selected. Here, although fitness is the reproduction of selectable individuals (units of reproduction), the manifestation of death ultimately lies at biochemical scales. This has led some to invoke oxidative stress as a primary agent for phylogenetic diversification and natural selection in terrestrial living systems (Rayner 1997, Watkins 1998, Watkins, Beeching, Rayner 1998 a, b, Rayner 1999, Rayner, Watkins & Beeching 1999). From considering the possible existence of universal feedback processes in living systems it can be deduced that the more the process of reciprocal exchange is overlooked or ignored, the more it is reasonable to regard life as mechanistically pre-determined by linear series of discrete genetic switches without relation to each other (Lewontin 1994, Rose 1997, Hurst 1997). By the same token, the more that organisms limit the potential for reciprocal environmental feedback, the more that the development of organisms does appear as pre-determined by the information from the past environment stored as inherited information. Layers of cuticle or skin and other homeostatic devices filter out much present contextual input. However, in this latter scenario, what genetic determinists do not appreciate is that the developmental feedback process with environment is still continuing here, just in different proportions to other life forms with less insulated boundaries.

It has been generally thought that most metazoan animals possess determinate development, not meaning necessarily that they were genetically pre-determined, though the two meanings were linked philosophically (Kordon 1993, Honderich 1999, Hook 1958). With determinate development, a cell type is permanently

fixed in its development so that once a cellular function is developed the cell can-not then change its developmental function or return to a totipotent state (Slack 1998). On the other hand, many microbes, plants and fungi possess indeterminate development. Here one particular developmental function can be phased to a different one in due course. So fungal development (chapter 1, figure 1.4), as opposed to metazoan animal and some plant development, maintains totipotency. Because of this one might think that fungi are different and perhaps less relevant to other fields in biology were it not for a highly conserved basic eukaryotic cellular ultrastructure (chapter 1, figure 1.5) (Havsteen 1997). It turns out that there are also exceptions to the rule that animals exhibit determined development. In the metazoan world are sea sponges and some species of hydra that maintain totipotency and can be separated to constituent cells and then re-form (Goodwin 1994 a, b). Also tails and limbs of some fish, amphibians and reptiles can re-develop if removed, and blood vessels, bones and some neurones of most animals grow in indeterminate branching patterns when viewed at appropriate scales (Losos *et al.* 2000). One of the best though macabre illustrations of mammalian cellular indeterminacy appears inside large tumours where partially developed organs of all kinds can often be found through a process of incremental loss of cellular identity. Also a similar phenomenon of metaplasia where a whole or partial tissue type will revert to a previous identity when the cell type is bathed in tissue fluid with abnormal environmental signals (for example from pancreatic tissue to liver tissue), after removal of vital metal ions (especially copper and zinc) (Bartoli & Bartoli 1984, Rao, Subbarao & Reddy 1986) or co-factors from the dietary environment of mammals (Aronovitch, Godinger, Sumuni, Czapski 1984). Such environmental constituents all happen to play a large role in signal mediated reciprocal developmental feedback processes. Metaplasia has been shown so far in rats, mice and humans (Slack 1992). It is possible and probable that proportions of saturated and unsaturated fats alter membrane properties in such a way as to mask and unmask active sites of cell membrane-bound proteins involved in cell signalling. This may also affect the potential for a membrane depolarisation to open membrane-bound ion-channels.

In the plant kingdom most cells, in certain environments, can exhibit totipotency, a feature used by plants to reproduce clonally (Wijesinghe & Hutchings 1997). Despite this, many terrestrial plants are classed as developmentally determinate because the same cell rarely naturally switches from one developed function such as being in a leaf, to others such as being in a root. Perhaps exceptions to pigeon-holed developmental categories add weight to ideas that a curtailed potential to develop certain ambits of reciprocal feedback is achieved by layers and enfoldings of membrane, cuticles and skin. These have evolved in animals since, in certain fixed relations between cells and tissues, totipotency represents a threat to the symmetry of the enclosed body plan and would be deleterious (causing tumours). I suggest that this raises the intriguing possibility that differences between developmental fixedness of cells may actually be more subtle and related to an organism's natural history and symmetry rather than being neatly categorised along taxonomic boundaries. This then increases the relevance of mycology to the biological sciences as a whole since fungi may teach us some hitherto missing component about the indeterminate development of life rather than be seen as a quirky evolutionary offshoot. The latter point is particularly relevant when considering that animals, with their enclosed and fixed body plans, represent less than half of the postulated diversity of life i.e. including extrapolations for fungi, bacteria and viruses (Wilson 1992 a, Hawksworth 1991,1998). Furthermore I suggest that perhaps the degree to which an organism is shielded in proportion to surface area from the outside world by layers of bark, skin or water may indicate the general degree of fixedness or "hard wiring" of its development. The reason to take surface area and volume into account when regarding the status of an organism's developmental determinism is easy to see given the following example. *C. elegans* is,

at the scale of an individual nematode worm, genetically pre-determined, with a small surface area to volume ratio. Consequently its skin presents a minimised interface to the outside environment for the volume of the organism contained within. On the other hand a common ink cap fungus *Coprinus picaceus* has a phenomenal surface area to volume ratio and thus a larger interface with the outside environment in order to assimilate. Consequently this fungus is developmentally more indeterminate since it has to utilise more information signals from the external environment to survive the changes taking place in it. Relatively speaking, the environment is more influential to the individual fungus than to the worm. This is especially so when considering that a substantial amount of fungal metabolism occurs outside its hyphae through nutritive catabolism, whereas the nutritive catabolism of the worm occurs internally within its alimentary tract. I think that this is a somewhat novel approach to the evolutionary significance of different surface area to volume ratios, having usually been used to study heat loss in relation to homeothermic mammals and ectothermic reptiles (Bonner 1965). I suggest that it may be possible to consider the continuous cycling of developmental feedback with respect to surrounding conditions across boundaries of varying shapes and degrees of permeability. In this scenario, developmental feedback across large surface areas of sensitive hyphal membrane (Lossel 1990) and cell wall, appears to enable woodland fungi to display a chemical versatility and mycelial boundary dexterity (Massey *et al.* 1999) which is highly sensitive, indeterminate and consequently more fluidly dynamic than that displayed by many large metazoan animals. Many animals have reduced surface area to volume ratios and thickened and desensitised their external boundaries. Fungi and the larvae which are incumbent within mycelia appear to have retained a reciprocious flexibility of development and genetic response, which suits their dynamic host arenas of decomposing substrates in which developmental feedback is exhibited (Rainey & Travisano 1998, Rainey 1991, Rayner & Todd 1982, Rayner *et al.* 1984, Lossick 1997).

Leaving microcosm to returning briefly to macrocosm, a thought stemming from the "interactivist perspective" I held during fieldwork (chapter 3), was that, as an ape walking under patchy tree cover, the height of woodland canopy rapidly changed between approximately waist-height and 20 metres in height. The scale of the ape continually changes in relation to canopy-height and size of vegetation architecture. As these changes occur, so too should the methods by which ecologists relate to canopy-cover and other vegetation (Chazden 1988, Deutchman, Levin & Palaca 1999). This requires novel units of measurement, relative to the succession of multicellular and ecological communities themselves (Smil 2000), and not to discrete metric standards. This relativist perspective need not complicate but may enhance future investigations of multiply overlapping factors that influence emergent patterns of morphology at many scales (Sparrow 1999a). Seeing ecosystems simultaneously through many scales also enables comparisons to be made between taxa in terms of general principles of system-evolution, adaptation and development (Petraitis & Latham 1999, Sparrow 1999b). These comparisons involve the confluence of interactive-symmetry-affecting-processes (Moller & Swaddle1997) which produce thermodynamically ergodically-stable manifolds (Osserman 1986, Strogatz 1994). Thermodynamic, fluid-membranous breaking of symmetry produces emergent biological patterns such as networks which expand under contraction via assimilation, branching, anasomosis, distribution, recycling and reproduction (Rayner, Watkins & Beeching 1999, Rayner 1997). These occur through alterations of boundary insulation which allow the system to remain coherent as it receives inputs of potentially disruptive energy (Rayner 1997, Rayner *et al.* 1999 b, Stanton, Roy & Thiede 2000).

When comparing relative amounts of boundary thickness between taxa in this way, some interesting thoughts come to mind. For example, perhaps plants and fungi could not physically possess the high surface area to volume ratios through which they forage efficiently for energy and nutrients if they were constantly living within tough and stable walls (Riedler 1978). The assimilative regions of an organism with high surface areas that forage without the physical protection of thick external body skins are, however, partial to the full pattern of heterogeneous and unstable environmental dynamics that often disturb the relative positions and thus break the symmetry of roots and hyphae. So plants and especially fungi have perhaps evolved the art of indeterminacy to counter potential disruption to their coherence and therefore have obtained a flexibility in development in through conserving their ability to forage and extend themselves through heterogeneous sources of nutriment (Smith 1999). Conversely, animals have evolved outside-in by assimilating only through internally-held, sable albeit high surface-area gut membranes. From the outside of the animal it appears as if insulated organisms approach closed-systems, variably insulated from disruptive effects caused by continuous external environmental changes. A disadvantage of this evolutionary-mode prevents animals from simply assimilating to derive nutrition from their environment. Instead, animals are forced to actively locomote from place to place to direct nutriment into their assimilative centres, and whilst they forage for food, they risk exhausting their energy reserves. Insects, in stark contrast to fungi, must therefore waste considerable energy in physically moving their guts to make contact with sources of food. Plants and fungi on the other hand, which viewed from outside the organism, approach open-systems, always assimilate nutrients at multiple-sites and develop variable responses to different quantities of nutrition and stress. The parallels between development of both types of metazoan taxa thus become most strikingly clear at two scales: first when comparing the views of animal-insides, blood-vessels, neurones, tissues and cell-types, and second when comparing the foraging or migration trajectories of whole animal behaviours in time-lapse or large group dynamics such as fish-shoaling, goat and wilder beast herds, army-ant raids, insect swarms, and human traffic-flows with the exploratory and distributive patterns of plants and fungi (Watts 1999, Couzin 1999, Franks 1996, Rayner & Franks 1987, Rayner *et al.* 1999 a, Rayner 1999). At these scales, living systems can be seen to be neither wholly open-, nor wholly closed-systems, but neatly sit in the middle with degrees of openness, fluidity, mani-foldedness and indeterminacy being displayed whenever the scales of view incorporate branches, anastomoses, adaptive networks or assimilative and distribution pathways of least resistance (Kroon & Hutchings 1994, Rayner 1997, Rayner *et al.* 1999 a).

These relativist concepts (Sparrow 1999 a,b) touch greatly on the issue of scale and fractal architecture in natural systems (Wilson 1992 a, Fielder & Ahouse 1992, Taylor 1993, 1994, Laurence 2000) and their relation to population patterns (Doebeli & Ruxton 1998), long-term dynamics (Dunnett, Willis, Hunt & Grune 1998), effects of population clumping (Doebeli & Jong 1999) habitat structure, heterogeneity, biogeography and geographic mosaic (Tivy 1971, Forman 1995, Thompson 1994).

Since wood itself provides potential accommodation in which so much invertebrate and microbial biodiversity can interact (Dajos 1974, 2000, Wilson 1992 a), the conservation value of woody debris, upright snags and living old hollow trees cannot be over-emphasised (Roper 1999, Nilsson, Hedin & Niklasson 2001, Ehnstrom 2001).

I hope that my thesis has highlighted the dynamic processes of nature that are intrinsic to the natural interactive states of ecological communities. I suggest that life-cycle stages of species with more indeterminate and interactive ambits, through their close proximity to others, possess a greater potential to exhibit co-habitative, site-specific, metabolic coupling, leading to new compounds formed at interaction interfaces (Whalley & Edwards 1995). This process appears to produce dynamic, synergetic structural and polymeric diversity leading to physical change in development of forms over an immense range of scales across nested boundaries from the metabolic to metazoan to meta population (Levin 1999, Levens 1968, Duarte, Boldrini & Dos Reis 1998, Hartshorn & Bynum 1999, Salvador 2000, Rayner 2000).

I suggest that **interactive states** of small-scaled ruderal organisms at the root of food-webs enhance proportions of **indirect effects** and **feedback** that form **adaptive networks** through reciprocal intra-dependent **synergies** across biotic and abiotic mani-folded interfaces. Such holey, microbial surfaces incorporate multiple-scaled fluid-affects which influence metabolic, epigenetic, genetic, phenotypic and environmental flux, which, in spreading over heterogeneous landscapes, tends to enhance diversity and symbiosis through feedback. This catalytic effect of **interactive-coupling** of cells, microbes and populations across varied surfaces in flux also realises the potential for ecological anabolism and increased survival of less common larger-scaled organisms and their complex assemblages. The emergent properties of such architecture in space are ones in which catabolic and competitive exclusion events are reduced. Perhaps it is knowledge of such interactivity in heterogeneous space that is our challenge to understand and apply in ecologically meaningful ways.

9. BIBLIOGRAPHY

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Appendix 1: Public understanding of science essay (1999)

BIO-DIVERSE SPICE AND CULINARY DELIGHTS

Cooking is perhaps like rotting; lots of bubbles are produced and systems turbulently change over time. Both processes create an a microcosmic architecture in which ingredients interact inside dynamic spaces, some fluid-filled and some not. In the forest, this form of architecture may enhance the potential for biodiversity to symbiotically co-exist rather than become dominated by just one competitive species.

Rotting, as the forest's major recycling process, is as much a part of biodiversity's nutriment as eating is to us. A surprising amount about emergence of patterns, biological development and how so many different species co-exist, can be found from the local study of small scale interactions in microcosm and even cooking a nice meal. Microbes live everywhere, interacting, inducing turbulence, hidden within and between all plants, inside trees, soil and even you. They are the millions of species of bacteria, fungi and slime moulds. Without these creatures life would never have left the soup-starter, neither would herbivores and millions of plant-eating insects be able to digest any leaves or wood and so carnivores would have no prey. I study how microbes interact with the decomposition processes in forests where they dwell in relation to each other and the environment. I combine observations from a local woodland, where I get pretty cold and wet, with experiments back in the 'insect-fungus lab' at Bath university. Underneath my desk are a pair of very muddy boots and the desk surface is littered with pieces of wood and bark in various states of decay.

It is underneath layers of rotting bark that I find a collection of interacting fungi of various colours and the tiny insect larvae that eat them. There is a whole world under the rotting bark of one hazel log with tiny branching patterns mirroring those of continental river systems, trees, nervous systems and the synaptic interconnections of the brain. Piles of coppiced hazel logs, created 5 years ago, have gradually rotted ever since allowing me to map and monitor their composition of insects and fungi. I am nearly at the end of my study and so are the hazel logs. I have grown fungi and insects in Petri dishes in the laboratory to observe their relationship closely. The insects affect fungi by grazing them and also by enhancing colour changes called "spalting" by carpenters who have used such wood for millennia to make decorated wooden bowels and plates to hold our meals. Unknown to most carpenters, spalting alters the outcome of fungal interactions within wood by marking territories so that each fungus is less able to oust another. Spalting raises the total number of different fungi and insects that can co-exist inside a rotting log. Indeed there is a feedback cycle whereby the interaction of insects with fungi increases spalting so that greater biodiversity exists than would otherwise be the case. Each wave of new migrant insect ingredients interact with resident fungi adding to previous "spalting", so increasing the colours that emerge chemically and give rise to wood-cavitation, the emergence of an architecture of inter-connected chambers in which insects can dwell. The fungi which produce the most colours appear to be offered symbiotic advantage by these events making attack by other fungi more difficult. In return, their spalting enhances the lifting of bark from logs. Lifted bark blisters provide an important cavity-matrix of spaces in which many insects and other invertebrates can dwell.

So rotting architecture emerges from a developmental coupling of the effects of insects and fungi on their woody environment and vice versa. The patterns observable have unintentionally self-organised from the ingredients in the environment, energy and life. In a similar way, the final taste of a meal is an outcome of a developmental process involving the combined effects of initial conditions such as potatoes, water and heat, and added ingredients, such as interactions between the taste of various herbs, vegetables and spices. However, in both cooking and rotting, the many synergistic interactions may also affect each other indirectly. Even if I measure out my ingredients exactly each time, the final taste and look of my meals remains about as chaotically predictable as the weather. Luckily this keeps things full of creative surprises.

Trunk call: Dr Peter Holmes on Bredon Hill, looking for signs of the violet click beetle Photograph: Russell Sac

Happy landing for jumping beetle

The Independant - November 1995

RICHARD SMITH

English Nature is set to spend £15,000 planting 300 trees to help save a rare jumping beetle from extinction in Britain.

The money will be used to preserve a Midlands beauty spot which inspired a poem by A E Housman, thereby making it a safe haven for the violet click beetle.

Experts fear that unless such action is taken, the tiny creature will eventually be wiped out on Bredon Hill in Hereford and Worcester – one of its two remaining breeding sites in England.

Until now the beetle has found an ideal home among the ancient ash, field maple, beach and oak trees growing on Bredon's hillside scrub and chalk grassland which has remained unploughed for centuries. But the beetle's lifeline – the next generation of mature open woodland – is almost non-existent. Many of the trees were old even at the end of the last century, when in *Bredon Hill* Housman wrote:

In summertime on Bredon
The bells they sound so clear;
Round both the shires they ring them
In steeples far and near,
A happy noise to hear

Dr Peter Holmes, English Nature's conservation officer for the region, said: "The violet click beetle is so rare that we know little about its lifestyle and the adult has only been seen five or six times. But it seems to live in a soup-like mixture where birds have nested, squirrels have died and fungus grows in hollow trees with leaf litter.

"So much modern woodland is too dense for these invertebrates, but Bredon Hill has been tree-covered for centuries and the landscape profile is the nearest thing we have to an ancient woodland habitat.

"There is also hawthorn on the hill, which provides nectar for these rare species to feed on and a place to meet and mate when they come out of the dead wood in spring.

"But the lack of young trees means the violet click beetle would certainly disappear from Bredon if it was not for the action we are taking now. They would only survive as long as the current generation of veteran ash."

Most of 380-hectare Bredon Hill is privately owned and English Nature has been working closely with farmers who use it for grazing cattle and sheep. Some ash are being pollarded to hasten maturity and violet click beetle larvae has been found on 17 trees.

Altogether 67 scarce species of beetles, including the rare blood red *ampedus rufipennis* have been found on the hillside.

The violet click beetle owes its name to the sound made by a spring-like appendage which enables it to leap a foot high and escape from predators.

The European Union lists the half-inch-long beetle – which is also found in Windsor Great Park – on a directive naming scarce species which are cause for international concern.

Rare breed: A close look at the violet click beetle

Appendix 2: Violet click beetle species recovery plan.

Appendix 2: Violet click beetle species recovery plan meeting.

Violet Click Beetle Species Recovery Action Plan Meeting
Windsor meeting Thursday 11th January 1996

Present

Ted Green - EN,
Keith Alexander - NT
Howard Mendell - Ipswich museum curator
Alan Rayner - Bath Uni
Stuart Reynolds - Bath Uni
Roger Guevara - Bath Uni
Christian Taylor - Bath Uni

Outline of day's proceedings:
Met at Windsor park in morning and went straight to field sites to examine habitat of Limoniscus violaceus . At first site examined the artificial hollow beech which Ted had set up next to old base of beech tree where L.violaceus found. Also looked around that area in other possible locations to get a feel for where beetle not likely to be found. Then off to second and third sites where examined similar habitat on old hollow beech trees elsewhere in park to get a good feel for were and were not to find the species. Had lunch in pub and continued talking about matters relating to conservation of L.violaceus and in general before re-convening at Silwood Park Imperial College to brainstorm on the species recovery action plan.

Mycological observations:
Pseudo sclerotial plate honeycombe structures seemed a common factor associated with habitat. Fragments of psp found both in the dark wood mulch in which the insects reside and also often hanging above or lining wood mulch in the internal cavity of hollowing tree. Two species of fungus principally involved in psp formation ; basidiomycete Armilaria mellia (honey fungus) - leaving characteristic rhizomorphs and ascomycete Astulina deusta - leaving characteristic brittle black pustular fruiting bodies.

Brainstorming Session towards conservation guidelines.
Broad objective to study ecology and conserve rare habitat in UK, perhaps with a view to releasing L. violaceus into UK habitats in the future. Also to provide habitat in the future by determining the niche space formation processes and facilitating these at a national level.At the broadest level the issue becomes old tree conservation. The recovery action plan must set out to promote the conservation of old trees - even if rotten and or hollow. To promote the conservation value of old trees with huge numbers of micro and macro niches on and inside their surface areas on which large numbers of species are dependant.
At one time we may assume that the population of L.violaceus covered the whole or a substantial part of Southern Britain. At present L.violaceus is a relic species in distinct populations in remains of ancient forest in which the many conditions necessary for its habitat are found. At Brenden hill (Cotswold scarp) this habitat is found mainly in hollowed out pollarded oaks of considerable age (see Independant article 5th January '96 pp7) whereas the conditions fulfilling the niche requirements at Windsor happen to be in old hollowed beech. Other sites with many ancient hollow trees in S. Britain include scarp vale of Evesham, Ashridge, Horner woods, McCormic in Cornwall and

Densil's waste near Plymouth. Also there are many hollowed trees which are being felled all over S. Britain at the moment for a variety of reasons. Most tree clearance at present is due to road building. In a natural forest it would be interesting to know what proportion of trees are being actively hollowed or are hollow and providing wood mulch, either standing or on forest floor. Is it as high as 50%? If so then woodland over-clearance and tidying could be causing habitat to dwindle in UK due to a relative lack of rotting wood both high in trees, dead standing trunks or at ground level. Bat and wood pigeon scat seem an important factor to provide nitrogen sources in the wood mulch. Cavities in trees and the processes that lead to them can be seen as vital for conservation purposes. We need to increase our understanding of the nature of trees as ecosystems - especially once they start hollowing. Studies must be done to determine the complex processes of niche compartmentalisation, diversification and succession on and within hollowing trees. Do different trees hollow in different ways? How are final niche dimensions dependant on tree, insect, fungus and animal interrelationships? What about effect of micro-climate? The wood mulch seems very specific; how wet?, how dry?, how sheltered?, how large a mass required to support populations?, how stable in abiotic factor fluctuations compared to external ambient climate?, how relevant is pH of wood mulch?, if the beetles are omnivores, what species are they feeding on in wood mulch? how many other species (biomediates) need to be present in wood mulch? are there indicator species? what roles do fungi and fungal pseudo sclerotial plates play?, is there a molecular attractant that allows the beetle to hone in to the right wood mulch? Another possibility is that the wood mulch with fungal psp is devoid of chemical toxins found elsewhere in rotting wood since psp acts rather like activated charcoal and sequesters aromatics and free radicals into the black melanin cross-bridges, thus rendering surrounding woody debris innocuous. This follows since the beetles are very chemically sensitive. They dislike harsh chemical environments. Could L.violaceus effectively hone into spaces in olfactory sensory landscapes devoid of toxic aromatics - thereby finding niche? The necessity for the wood mulch to be both innocuous with psp and also fitting a wide range of micro-climatic and shelter conditions could be what determines the rarity of the habitat. Innocuous things are not common. It may also be useful to bare in mind the evolutionary line of the insect comes from ground level soil/compost - living beetles then adapting to live in similar habitats within trees where composting also occurs.

Conclusions:
Sheltered habitat with particular kinds of fungi and insects present in compost.
i) Advise to leave dead trees standing wherever possible.
ii) Advise to leave mature trees to develop habitat required.
iii) Advise to cater for future short fall in middle aged and mature trees by planting and conservation of intermediate-aged trees especially oak, ash and beech but also others where L.violaceous found.
iv) Set up Saproxylic interest group - perhaps with link to Ancient Tree Forum?

Appendix 4: Fishers' Exact Test for log pile data (SPSS for windows)

CROSS TABULATION
CECIDS vs PSP

CECIDS * PSP Crosstabulation

			PSP		
			.00	1.00	Total
CECIDS	.00	Count	5	2	7
		Expected Count	1.7	5.3	7.0
	1.00	Count	1	17	18
		Expected Count	4.3	13.7	18.0
Total		Count	6	19	25
		Expected Count	6.0	19.0	25.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	11.990[b]	1	.001		
Continuity Correction[a]	8.651	1	.003		
Likelihood Ratio	11.454	1	.001		
Fisher's Exact Test				.002	.002
Linear-by-Linear Association	11.510	1	.001		
N of Valid Cases	25				

SIGNIFICANT (P<0.005)

a. Computed only for a 2x2 table

b. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 1.68.

Symmetric Measures

		Value	Asymp. Std. Error[a]	Approx. T[b]	Approx. Sig.
Nominal by Nominal	Phi	.693			.001
	Cramer's V	.693			.001
Interval by Interval	Pearson's R	.693	.163	4.604	.000[c]
Ordinal by Ordinal	Spearman Correlation	.693	.163	4.604	.000[c]
N of Valid Cases		25			

a. Not assuming the null hypothesis.

b. Using the asymptotic standard error assuming the null hypothesis.

c. Based on normal approximation.

APPENDIX 5 : Insect emergence trap raw data - 1998-1999

TRAP description: DATE:	Trap 1 + Liquorice (Hazel coppice pole by lane)	Trap 2 + Liquorice (log pile 2 plot one)	Trap 3 (log pile 5 plot 2)	Trap 4 (Hazel coppice pole by path plot 2)	Trap 5 (small log pile plot 1)	Trap 6 + Liquorice (Hazel coppice pole near plot 1)	Trap 7 (horizontal attatched hazel branch)	Trap 8 + Liquorice (upright hazel log against tree)	Trap 9 (Wood pile 33 near path)	Trap 10 (Wood pile 34 near path)
APRIL	1 spider 1 cecid	1 beetle 1 cecid 1 sciarid	1 beetle 2 mycetophilid	1 wasp 1 bettle 1 fly	1 sciarid		1 beetle (predatary) (*Otiorhynchus clavipes*)		1 sciarid	
MAY	1 midge 4 cecid 2 spider 2 predatary diptera	3 sciarid 1 cecid 1 beetle 2 wasp 2 spider 1 aphid	4 beetle 1 spider	1 cecid 1 sciarid	4 sciarid 2 cecid 3 beetle 2 earwig	1 mycetophilid 1 wasp 2 spider 1 cecid 5 sciarid	4 cecid 3 sciarid 2 wasp 1 earwig	2 sciarid 1 beetle	1 cecid 4 sciarid 2 diptra 3 spiders 1 moth	1 cecid 1 sciarid 1 spider
JUNE	2 earwig 1 spider 1 weevle 1mycetophilid	2 cecid 1 parasitic wasp (*Aluacus striatus*) 1 beetle 1 spider (*Atypus affinis*)	6 earwigs	1 earwig 1 beetle 2 damsel bugs (*Redvildae sp.*) 1 spider	2 earwig 1 parasitic wasp (*Aluacus sp.*) 1 mycetophilid 2 beetles 1 large diptera			1 earwig 1 myceto-phillid 1 large diptera 1 beetle 1 cecid	1 parasitic wasp (*Aluacus sp.*) 1 spider 1 earwig	
JULY			2 cecid 5 sciarid 2 mites 3 large diptera 1 weevle 1 wasp 1 gall wasp			4 sciarids 2 cecids	1 mycetophilid 2 sciarids 2 cecids 1 wasp 1 spider 1 beetle		3 cecids 5 sciarid 1 spider 1 beetle (*Byrrhus sp.*)	

JULY continued	Trap 1	Trap 2	Trap 3	Trap 4	Trap 5	Trap 6	Trap 7	Trap 8	Trap 9	Trap 10
			1 mycetophilid 2 bark beetle 4 aphids 8 beetles 1*Rhagonycha fulva*				2 bloody-nose beetle			
OCT	4 cecid 1 predator *Lissonota sp.* wasp 3 earwig	1 spider	30 earwig 7 sciarid 3 cecid 1 beetle 10 mites 2 parasitic wasp (*Agriotypus sp Aulacus sp*) 1 ladybird 1 lacewing larva (*Chrysopa sp.*) 1 beetle (*Lyctus sp*) 2 *Trypoxylon figulus*	3 sciarid 2 cecid 2 earwig	1 mycetophilid 1 cecid 4 earwig 4 beetle	1 parasitic wasp (*Aulacus striatus*)	1 cecid 1 weevle (*Otiorhynchus clavipes*) 1 beetle 1 ladybird 1 mycetophilid 3 earwig 1 drosophila 3 spider	4 earwig		2 earwig 1 *Bibio marci* 1 *Hilara maura*
NOV	1 beetle			1 earwig 4 beetle	1 earwig 2 beetle 1 sciarid			1 cecid 1 earwig	1 sciarid	
DEC		1 parasitic wasp (*Gasterupton sp.*) 5 spider 1 beetle	1 sciarid 3 earwig 1 wasp (*Mymar sp.*) 1 ladybird 3 spider 1 mycetophilid 1 Phorid			3 midge (*Chaeoporus sp.*) 4 cecids 8 sciarids	1 cecid 1 death watch beetle (Xestobium sp.)			

DEC continued			1 parasitic wasp (*Aluacus sp.*)							
JAN										
FEB FEB (continued from first sheet)	5 collembola 2 spiders 1 cecid 1 ladybird larva 1 earwig 1 lacewing larva (predatory)	1 earwig	1 cecid 2 spiders 8 earwig	1 earwig		3 lacewing larva 4 *Sylvicola sp.* 1 *Oscinella sp.*	2 beetle 1 cecid		2 earwig	1 earwig 1 diptera (*Pericoma fuliginosa*)
MAR		1 ladybird 2 cecids 1 sciarid 4 spiders 1 aphid 1 parasitic wasp (*Aluacus sp.*) 1 Phorid fly (*Phora atra*)	1 spider 1 beetle 1 parasitic wasp (*Aluacus sp.*) 4 *Leptura guttata*	1 cecic 1 sciarid 3 earwig 4 midges (Culicoides sp.) 1 Otiorhynchus clavipes	2 cecids 5 sciarid 3 beetles 2 earwig					
MAY	*Dioctria sp.* (robber fly) 4 cecids 4 spiders	2 beetle (*Otiorhynchus clavipes*) 2 small beetle 1 spider	20 earwig 5 beetle (*Otiorhynchus clavipes*) 1 ladybird 5 beetle 3 spider 1 cecid 1 ant	2 earwig 1 sciarid	2 annelida worms 13 small beetle 11 earwig 4 spider 1 *Pericoma fuliginosa* 1 *Lonchaea chorea*	1 *Suillia variagata* 1 earwig 1 *Periphredon lugrubis* 1 *Biorhiza sp.*	1 cecid 1 spider (*Lissonota sp.*) 1 parasitic wasp (*Aluacus sp.*)	4 spider 2 cecids 1 gall wasp (*Phariasis sp*) 1 micro moth (*Pandemis sp.*)		

MAY Continued			2 wasp - tiny parasitic (*Pemphredon sp.*)		1 *Lymexylon navale* 1 *Mymar pulchellus* 1 *Ophion sp.* 4 *Doomius maculatus* 1 *Agriotypus sp.* 1 *Phora atra*					
AUG			5 earwig 4 spider 8 small beetle 2 wood beetle 5 other beetle (*Tomocerus sp*) 1 saw fly (*Xiphydria sp*) 1 parasitic wasp - metalic (*Torymus sp.*) 1 parasitic wasp (*Agriotypus sp.*) 1 lacewing larva *Suillia variegata* *Phora ata* 1 parasitic wasp (*Pteromalus sp*) 2 tiny parasitic wasp (*Mymar sp.*) 1 gall wasp		2 spiders 1 moth (*Acronicta sp.*) 1 *Otiorhynchus sp.* 1 *Lissonota setosa* 7 woodworm (*Anobium sp.*) 2 sciarid 4 cecid		34 woodworm beetle (*Anobium sp.*) 4 spider 1 earwig 4 cecid (*Paederus sp.*) 4 beetle 1 gall wasp (*Diplolepis sp.*) 1 parasitic wasp (Aluacus sp.) 1 sciarid		17 *Otiorhynchus sp.* 3 earwig 1 moth (*Acronita sp.*) 4 spider 30 tiny beetle 1 *Phora atra* 2 parasitic wasp (*Aluacus sp.*) 1 cecid 1 beetle (*Philouthus sp.*) 3 *Platyura marginata* 2 Drosophila sp. 2 Tiny wasps (*Culicoides sp.*) 2 *Oscinella frit*	2 *Otiorhynchus sp.* 31 small beetle 10 large beetle 20 juvenile spider 1 *Phora ata* 1 lacewing larva 1 *Agriotypus sp.* 2 *Pteromulus sp.* 12 earwig *Anthomomus sp.* 1 cecid 1 gall wasp *Cynips sp.*

	Trap 1	Trap 2	Trap 3 (*Phanacis sp.*)	Trap 4	Trap 5	Trap 6	Trap 7	Trap 8	Trap 9	Trap 10
AUG continued									1 *Suillia sp* 1 *Platyursa sp* 1 *Empis sp* 1 *Cerotelion sp.* 1 weevle (*Anthonomus sp.*) parasitic wasps: 1 *Aluacus sp.* 2 *Agriotypus sp.* 1 *Mymar sp.*	
TOTALS	44	43	200	36	105	43	90	27	106	91

TYPES OF CECID CAUGHT IN FIELD TRAPS

TRAP	TRAP 4	OTHER TRAPS	TRAP 10
Type of cecidomyiidae found	Lestremiinae sp.	Heteropezinae sp. Lestrimiinae sp. + several non paedogenic genera	Heteropezula sp.

Appendix 5 pp 5

Appendix 6: Pure culture area statistics - image analysis (Minitab for windows).
PURE CULTURE AREAS (cm^2)

Hypoxylon fuscum PURE CULTURE (top views) (n=9)

Mann-Whitney Confidence Interval and Test

```
Deeply M   N =   9     Median =      7598.8
Deeply M   N =   9     Median =       345.5
Point estimate for ETA1-ETA2 is      7253.3
95.8 Percent CI for ETA1-ETA2 is (1286.5,14344.5)
W = 126.0
Test of ETA1 = ETA2  vs  ETA1 not = ETA2 is significant at 0.0004
SIGNIFICANT
```

H. fuscum top orientation (n=9) P< 0.001

Mann-Whitney Confidence Interval and Test

```
Melanise   N =   9     Median =       10743
Melanise   N =   9     Median =       19640
Point estimate for ETA1-ETA2 is       -8898
95.8 Percent CI for ETA1-ETA2 is (-15252,-2565)
W = 45.0
Test of ETA1 = ETA2  vs  ETA1 not = ETA2 is significant at 0.0004
SIGNIFICANT
```

P< 0.001

Deeply M = deeply melanised (almost black)
Melanise = green coloured

Hypoxylon fuscum PURE CULTURE - UNDERSIDE

(n=8)

Mann-Whitney Confidence Interval and Test

```
Deeply M   N =   8     Median =      2344.6
Deeply M   N =   8     Median =       512.1
Point estimate for ETA1-ETA2 is      1756.4
95.9 Percent CI for ETA1-ETA2 is (863.1,2283.9)
W = 98.0
Test of ETA1 = ETA2  vs  ETA1 not = ETA2 is significant at 0.0019
SIGNIFICANT
```

P< 0.005

Mann-Whitney Confidence Interval and Test

```
Melanise   N =   8     Median =       15149
Melanise   N =   8     Median =       13049
Point estimate for ETA1-ETA2 is        2923
95.9 Percent CI for ETA1-ETA2 is (-329,6633)
W = 84.0
Test of ETA1 = ETA2  vs  ETA1 not = ETA2 is significant at 0.1036

Cannot reject at alpha = 0.05
NOT SIGNIFICANT
```

Mann-Whitney Confidence Interval and Test

```
Hf +c      N =   8     Median =       17178
Hf con     N =   8     Median =       19986
Point estimate for ETA1-ETA2 is       -2758
95.9 Percent CI for ETA1-ETA2 is (-6727,-1539)
W = 40.5
Test of ETA1 = ETA2  vs  ETA1 not = ETA2 is significant at 0.0046
The test is significant at 0.0025 (adjusted for ties)
SIGNIFICANT
```

P< 0.005

Hf = area of whole *H. fuscum* colony. Deeply M = deeply melanised (almost black)
Melanise = green coloured

Hypoxylon fuscum PURE CULTURE WITH MEMBRANE (top views)

(n=9)

Mann-Whitney Confidence Interval and Test

```
Deeply M   N =   9     Median =      3479.0
Deeply M   N =   9     Median =       306.0
Point estimate for ETA1-ETA2 is      3173.0
95.8 Percent CI for ETA1-ETA2 is (1808.0,3740.1)
W = 126.0
Test of ETA1 = ETA2  vs  ETA1 not = ETA2 is significant at 0.0004
The test is significant at 0.0004 (adjusted for ties)
SIGNIFICANT
```

P< 0.001

Mann-Whitney Confidence Interval and Test

```
Melanise   N =   9     Median =      3752.0
Melanise   N =   9     Median =      7771.0
Point estimate for ETA1-ETA2 is     -4236.4
95.8 Percent CI for ETA1-ETA2 is (-7073.9,-1166.2)
W = 48.0
Test of ETA1 = ETA2  vs  ETA1 not = ETA2 is significant at 0.0011
SIGNIFICANT
```

P< 0.005

Mann-Whitney Confidence Interval and Test

```
Hf +c      N =   9     Median =       6536
Hf con     N =   9     Median =      19986
Point estimate for ETA1-ETA2 is     -13450
95.8 Percent CI for ETA1-ETA2 is (-15158,-12034)
W = 45.0
Test of ETA1 = ETA2  vs  ETA1 not = ETA2 is significant at 0.0004
The test is significant at 0.0002 (adjusted for ties)
SIGNIFICANT
```

P< 0.001

Hypoxylon fuscum PURE CULTURES WITH MEMBRANE - UNDERSIDE

Mann-Whitney Confidence Interval and Test

(n=9)

```
Deeply M   N =   9     Median =      3586.8
Deeply M   N =   9     Median =       298.0
Point estimate for ETA1-ETA2 is      3136.0
95.8 Percent CI for ETA1-ETA2 is (911.0,4736.7)
W = 126.0
Test of ETA1 = ETA2  vs  ETA1 not = ETA2 is significant at 0.0004
SIGNIFICANT
```

P< 0.001

Mann-Whitney Confidence Interval and Test

```
Melanise   N =   9     Median =       814.0
Melanise   N =   9     Median =      5577.6
Point estimate for ETA1-ETA2 is     -4429.0
95.8 Percent CI for ETA1-ETA2 is (-5361.0,-2411.9)
W = 51.0
Test of ETA1 = ETA2  vs  ETA1 not = ETA2 is significant at 0.0027
SIGNIFICANT
```

P< 0.005

Hf = area of whole *H. fuscum* colony.

Deeply M = deeply melanised (almost black)
Melanise = green coloured

PURE CULTURES AMALGAMATED DATA FROM IMAGE ANALYSIS (n=34)

Paired T-Test and Confidence Interval

Paired T for Deeply Melanised + c - Deeply Mel CON

	N	Mean	StDev	SE Mean
Deeply M	34	4020	3577	614
Deeply M	34	353	244	42
Difference	34	3667	3556	610

95% CI for mean difference: (2426, 4908)
T-Test of mean difference = 0 (vs not = 0): T-Value = 6.01 P-Value = 0.000
SIGNIFICANT

P< 0.001

Paired T-Test and Confidence Interval

Paired T for Melanised + c - Mel CON

	N	Mean	StDev	SE Mean
Melanise	34	7174	6072	1041
Mel CON	34	10772	5936	1018
Difference	34	-3598	5852	1004

95% CI for mean difference: (-5640, -1556)
T-Test of mean difference = 0 (vs not = 0): T-Value = -3.59 P-Value = 0.001
SIGNIFICANT

P< 0.005

(n = 34)

Paired T-Test and Confidence Interval

Paired T for Agar + c - Agar CON

	N	Mean	StDev	SE Mean
Agar + c	34	1300	2262	388
Agar CON	34	8832	5982	1026
Difference	34	-7532	6847	1174

95% CI for mean difference: (-9921, -5143)
T-Test of mean difference = 0 (vs not = 0): T-Value = -6.41 P-Value = 0.000
SIGNIFICANT

P< 0.001

Paired T-Test and Confidence Interval

Paired T for Hf + c - Hf CON

	N	Mean	StDev	SE Mean
Hf + c	34	11194	6247	1071
Hf CON	34	19954	167	29
Difference	34	-8761	6237	1070

95% CI for mean difference: (-10937, -6584)
T-Test of mean difference = 0 (vs not = 0): T-Value = -8.19 P-Value = 0.000
SIGNIFICANT

P< 0.001

Agar = clear agar with no mycelium
Hf = area of whole *H. fuscum* colony.

Deeply M = deeply melanised (almost black)
Melanise = green coloured

Vuilleminia commedens PURE CULTURES (top views)

(n=6)

Mann-Whitney Confidence Interval and Test

```
Insulate   N =   6     Median =      6839.0
insulate   N =   6     Median =      5566.5
Point estimate for ETA1-ETA2 is      1387.5
95.5 Percent CI for ETA1-ETA2 is (54.1,3750.6)
W = 53.0
Test of ETA1 = ETA2  vs  ETA1 not = ETA2 is significant at 0.0306
SIGNIFICANT
```

P< 0.05

Mann-Whitney Confidence Interval and Test

```
Diffuse    N =   6     Median =       12850
diffuse    N =   6     Median =       14387
Point estimate for ETA1-ETA2 is       -1536
95.5 Percent CI for ETA1-ETA2 is (-3568,975)
W = 30.0
Test of ETA1 = ETA2  vs  ETA1 not = ETA2 is significant at 0.1735

Cannot reject at alpha = 0.05
NOT SIGNIFICANT
```

P>0.05

PURE CULTURE Vuilleminia Commedens; COMPILLATION (n=6)

Mann-Whitney Confidence Interval and Test

```
Insulate   N =   6     Median =      6839.0
Insulate   N =   6     Median =      5566.5
Point estimate for ETA1-ETA2 is      1387.5
95.5 Percent CI for ETA1-ETA2 is (54.1,3750.6)
W = 53.0
Test of ETA1 = ETA2  vs  ETA1 not = ETA2 is significant at 0.0306

SIGNIFICANT
```

P< 0.05

Mann-Whitney Confidence Interval and Test

```
Diffuse    N =   6     Median =       12850
Diffuse    N =   6     Median =       14387
Point estimate for ETA1-ETA2 is       -1536
95.5 Percent CI for ETA1-ETA2 is (-3568,975)
W = 30.0
Test of ETA1 = ETA2  vs  ETA1 not = ETA2 is significant at 0.1735

Cannot reject at alpha = 0.05
NOT SIGNIFICANT
```

P>0.05 !

Insulate = insulated mycelium

Diffuse = diffuse assimilative mycelium.

Appendix 7: Combined culture foraging statistics (Minitab for windows).

Field cecid trajectory radii on Pure Cultures. **FORAGING LOOP RADII (mm)**

Paired T-Test and Confidence Interval

Paired T for Vc pure - Hf pure **(n=22)**

	N	Mean	StDev	SE Mean
Vc pure	22	8.059	4.442	0.947
Hf pure	22	5.159	2.122	0.452
Difference	22	2.900	2.776	0.592

95% CI for mean difference: (1.669, 4.131)
T-Test of mean difference = 0 (vs not = 0): T-Value = 4.90 P-Value = 0.000
SIGNIFICANT

P< 0.001

Paired T-Test and Confidence Interval

Paired T for Ln Vc pure - Ln Hf pure

	N	Mean	StDev	SE Mean
Ln Vc pu	22	1.949	0.542	0.115
Ln Hf pu	22	1.546	0.467	0.100
Difference	22	0.4035	0.1743	0.0372

95% CI for mean difference: (0.3262, 0.4807)
T-Test of mean difference = 0 (vs not = 0): T-Value = 10.86 P-Value = 0.000
SIGNIFICANT

P< 0.001

COMBINATION CULTURE Trajectory radii:

Paired T-Test and Confidence Interval

Paired T for psp - Vc

	N	Mean	StDev	SE Mean
psp	66	1.961	0.617	0.076
Vc	66	6.491	1.795	0.221
Difference	66	-4.530	1.273	0.157

95% CI for mean difference: (-4.843, -4.217)
T-Test of mean difference = 0 (vs not = 0): T-Value = -28.90 P-Value = 0.000
SIGNIFICANT

P< 0.001

Paired T-Test and Confidence Interval

Paired T for Hf - Vc

	N	Mean	StDev	SE Mean
Hf	66	4.586	0.628	0.077
Vc	66	6.491	1.795	0.221
Difference	66	-1.905	1.244	0.153

95% CI for mean difference: (-2.210, -1.599)
T-Test of mean difference = 0 (vs not = 0): T-Value = -12.44 P-Value = 0.000
SIGNIFICANT

P< 0.001

Paired T-Test and Confidence Interval

Paired T for Hf - psp

	N	Mean	StDev	SE Mean
Hf	66	4.5864	0.6275	0.0772
psp	66	1.9606	0.6169	0.0759
Difference	66	2.6258	0.1512	0.0186

95% CI for mean difference: (2.5886, 2.6629)
T-Test of mean difference = 0 (vs not = 0): T-Value = 141.07 P-Value = 0.000

P< 0.001

SIGNIFICANT

Comparison Trajectory radii between mono and combination cultures.

Paired T-Test and Confidence Interval

Paired T for Vc pure - Vc

	N	Mean	StDev	SE Mean
Vc pure	22	8.059	4.442	0.947
Vc	22	8.586	1.567	0.334
Difference	22	-0.527	3.014	0.643

95% CI for mean difference: (-1.864, 0.809)
T-Test of mean difference = 0 (vs not = 0): T-Value = -0.82 P-Value = 0.421
NOT SIGNIFICANT

P>0.05

Paired T-Test and Confidence Interval

Paired T for Hf pure - Vc

	N	Mean	StDev	SE Mean
Hf pure	22	5.159	2.122	0.452
Vc	22	8.586	1.567	0.334
Difference	22	-3.427	1.052	0.224

95% CI for mean difference: (-3.894, -2.961)
T-Test of mean difference = 0 (vs not = 0): T-Value = -15.29 P-Value = 0.000
SIGNIFICANT

P< 0.001

Paired T-Test and Confidence Interval

Paired T for psp - Vc pure

	N	Mean	StDev	SE Mean
psp	22	2.673	0.271	0.058
Vc pure	22	8.059	4.442	0.947
Difference	22	-5.386	4.225	0.901

95% CI for mean difference: (-7.259, -3.513)
T-Test of mean difference = 0 (vs not = 0): T-Value = -5.98 P-Value = 0.000
SIGNIFICANT

P< 0.001

Paired T-Test and Confidence Interval

Paired T for Hf pure - psp

	N	Mean	StDev	SE Mean
Hf pure	22	5.159	2.122	0.452
psp	22	2.673	0.271	0.058
Difference	22	2.486	1.869	0.398

95% CI for mean difference: (1.658, 3.315)
T-Test of mean difference = 0 (vs not = 0): T-Value = 6.24 P-Value = 0.000
SIGNIFICANT

P< 0.001

Paired T-Test and Confidence Interval

Paired T for Hf - Vc pure

	N	Mean	StDev	SE Mean
Hf	22	5.309	0.274	0.058
Vc pure	22	8.059	4.442	0.947
Difference	22	-2.750	4.192	0.894

95% CI for mean difference: (-4.608, -0.892)
T-Test of mean difference = 0 (vs not = 0): T-Value = -3.08 P-Value = 0.006
SIGNIFICANT

P< 0.05

Trajectaries of field cecids on Coreolus versicolor.

Paired T-Test and Confidence Interval

Paired T for pspDI/D3 - D1

	N	Mean	StDev	SE Mean
pspDI/D3	15	1.85	0.98	0.25
D1	15	22.29	20.75	5.36
Difference	15	-20.44	19.90	5.14

95% CI for mean difference: (-31.46, -9.42)
T-Test of mean difference = 0 (vs not = 0): T-Value = -3.98 P-Value = 0.001
SIGNIFICANT

P< 0.005

Paired T-Test and Confidence Interval

Paired T for pspD1/D2 - D1

	N	Mean	StDev	SE Mean
pspD1/D2	15	3.60	2.53	0.65
D1	15	22.29	20.75	5.36
Difference	15	-18.69	18.34	4.74

95% CI for mean difference: (-28.84, -8.53)
T-Test of mean difference = 0 (vs not = 0): T-Value = -3.95 P-Value = 0.001
SIGNIFICANT

P< 0.005

Paired T-Test and Confidence Interval

Paired T for pspD1/D4 - D1

	N	Mean	StDev	SE Mean
pspD1/D4	15	3.24	0.95	0.24
D1	15	22.29	20.75	5.36
Difference	15	-19.05	19.95	5.15

95% CI for mean difference: (-30.10, -8.00)
T-Test of mean difference = 0 (vs not = 0): T-Value = -3.70 P-Value = 0.002
SIGNIFICANT

P< 0.005

Paired T-Test and Confidence Interval

Paired T for pspDI/D3 - pspD1/D4

	N	Mean	StDev	SE Mean
pspDI/D3	15	1.850	0.976	0.252
pspD1/D4	15	3.240	0.946	0.244
Difference	15	-1.390	0.464	0.120

95% CI for mean difference: (-1.647, -1.133)
T-Test of mean difference = 0 (vs not = 0): T-Value = -11.61 P-Value = 0.000
SIGNIFICANT

P< 0.005

Paired T-Test and Confidence Interval

Paired T for pspD1/D2 - pspDI/D3

	N	Mean	StDev	SE Mean
pspD1/D2	15	3.600	2.531	0.654
pspDI/D3	15	1.850	0.976	0.252
Difference	15	1.750	1.667	0.430

95% CI for mean difference: (0.827, 2.673)
T-Test of mean difference = 0 (vs not = 0): T-Value = 4.07 P-Value = 0.001
SIGNIFICANT

P< 0.005

Paired T-Test and Confidence Interval

Paired T for D2 - D1

	N	Mean	StDev	SE Mean
D2	15	11.76	8.46	2.18
D1	15	22.29	20.75	5.36
Difference	15	-10.53	12.96	3.35

95% CI for mean difference: (-17.70, -3.35)
T-Test of mean difference = 0 (vs not = 0): T-Value = -3.15 P-Value = 0.007
SIGNIFICANT

P< 0.05

Paired T-Test and Confidence Interval

Paired T for pspD1/D4 - pspD1/D2

	N	Mean	StDev	SE Mean
pspD1/D4	15	3.240	0.946	0.244
pspD1/D2	15	3.600	2.531	0.654
Difference	15	-0.360	1.698	0.438

95% CI for mean difference: (-1.300, 0.580)
T-Test of mean difference = 0 (vs not = 0): T-Value = -0.82 P-Value = 0.425
NOT SIGNIFICANT

P>0.05

Paired T-Test and Confidence Interval

Paired T for pspD1/D2 - D2

	N	Mean	StDev	SE Mean
pspD1/D2	15	3.60	2.53	0.65
D2	15	11.76	8.46	2.18
Difference	15	-8.16	6.32	1.63

95% CI for mean difference: (-11.66, -4.66)
T-Test of mean difference = 0 (vs not = 0): T-Value = -5.00 P-Value = 0.000
SIGNIFICANT

P< 0.001

Paired T-Test and Confidence Interval

Paired T for pspDI/D3 - D2

	N	Mean	StDev	SE Mean
pspDI/D3	15	1.85	0.98	0.25
D2	15	11.76	8.46	2.18
Difference	15	-9.91	7.69	1.99

95% CI for mean difference: (-14.17, -5.65)
T-Test of mean difference = 0 (vs not = 0): T-Value = -4.99 P-Value = 0.000
SIGNIFICANT

P< 0.001

Paired T-Test and Confidence Interval

Paired T for pspD1/D4 - D2

	N	Mean	StDev	SE Mean
pspD1/D4	15	3.24	0.95	0.24
D2	15	11.76	8.46	2.18
Difference	15	-8.52	7.67	1.98

95% CI for mean difference: (-12.77, -4.27)
T-Test of mean difference = 0 (vs not = 0): T-Value = -4.30 P-Value = 0.001
SIGNIFICANT

P< 0.005

Paired T-Test and Confidence Interval

Paired T for D3 - D1

	N	Mean	StDev	SE Mean
D3	15	6.54	6.22	1.61
D1	15	22.29	20.75	5.36
Difference	15	-15.75	14.89	3.84

95% CI for mean difference: (-23.99, -7.50)
T-Test of mean difference = 0 (vs not = 0): T-Value = -4.10 P-Value = 0.001
SIGNIFICANT

P< 0.005

Paired T-Test and Confidence Interval

Paired T for pspDI/D3 - D3

	N	Mean	StDev	SE Mean
pspDI/D3	15	1.85	0.98	0.25
D3	15	6.54	6.22	1.61
Difference	15	-4.69	5.42	1.40

95% CI for mean difference: (-7.69, -1.69)
T-Test of mean difference = 0 (vs not = 0): T-Value = -3.35 P-Value = 0.005
SIGNIFICANT

P< 0.05

Paired T-Test and Confidence Interval

Paired T for pspD1/D2 - D3

	N	Mean	StDev	SE Mean
pspD1/D2	15	3.60	2.53	0.65
D3	15	6.54	6.22	1.61
Difference	15	-2.94	3.90	1.01

95% CI for mean difference: (-5.10, -0.78)
T-Test of mean difference = 0 (vs not = 0): T-Value = -2.92 P-Value = 0.011
SIGNIFICANT

P<0.05

Paired T-Test and Confidence Interval

Paired T for pspD1/D4 - D3

	N	Mean	StDev	SE Mean
pspD1/D4	15	3.24	0.95	0.24
D3	15	6.54	6.22	1.61
Difference	15	-3.30	5.36	1.38

95% CI for mean difference: (-6.27, -0.33)
T-Test of mean difference = 0 (vs not = 0): T-Value = -2.38 P-Value = 0.032
SIGNIFICANT

P<0.05

Paired T-Test and Confidence Interval

Paired T for D2 - D3

	N	Mean	StDev	SE Mean
D2	15	11.76	8.46	2.18
D3	15	6.54	6.22	1.61
Difference	15	5.220	2.933	0.757

95% CI for mean difference: (3.596, 6.844)
T-Test of mean difference = 0 (vs not = 0): T-Value = 6.89 P-Value = 0.000
SIGNIFICANT

P< 0.001

Paired T-Test and Confidence Interval

Paired T for D4 - D1

	N	Mean	StDev	SE Mean
D4	15	17.97	8.22	2.12
D1	15	22.29	20.75	5.36
Difference	15	-4.32	14.46	3.73

95% CI for mean difference: (-12.33, 3.69)
T-Test of mean difference = 0 (vs not = 0): T-Value = -1.16 P-Value = 0.267
NOT SIGNIFICANT

P>0.05

Paired T-Test and Confidence Interval

Paired T for pspDI/D3 - D4

	N	Mean	StDev	SE Mean
pspDI/D3	15	1.85	0.98	0.25
D4	15	17.97	8.22	2.12
Difference	15	-16.12	7.27	1.88

95% CI for mean difference: (-20.14, -12.09)
T-Test of mean difference = 0 (vs not = 0): T-Value = -8.59 P-Value = 0.000

P< 0.001

SIGNIFICANT

Paired T-Test and Confidence Interval

Paired T for pspD1/D2 - D4

	N	Mean	StDev	SE Mean
pspD1/D2	15	3.60	2.53	0.65
D4	15	17.97	8.22	2.12
Difference	15	-14.37	5.96	1.54

95% CI for mean difference: (-17.67, -11.06)
T-Test of mean difference = 0 (vs not = 0): T-Value = -9.33 P-Value = 0.000
SIGNIFICANT

P< 0.001

Paired T-Test and Confidence Interval

Paired T for pspD1/D4 - D4

	N	Mean	StDev	SE Mean
pspD1/D4	15	3.24	0.95	0.24
D4	15	17.97	8.22	2.12
Difference	15	-14.73	7.39	1.91

95% CI for mean difference: (-18.82, -10.64)
T-Test of mean difference = 0 (vs not = 0): T-Value = -7.72 P-Value = 0.000
SIGNIFICANT

P< 0.001

Paired T-Test and Confidence Interval

Paired T for D2 - D4

	N	Mean	StDev	SE Mean
D2	15	11.76	8.46	2.18
D4	15	17.97	8.22	2.12
Difference	15	-6.21	5.40	1.39

95% CI for mean difference: (-9.20, -3.22)
T-Test of mean difference = 0 (vs not = 0): T-Value = -4.45 P-Value = 0.001
SIGNIFICANT

P< 0.001

Paired T-Test and Confidence Interval

Paired T for D3 - D4

	N	Mean	StDev	SE Mean
D3	15	6.54	6.22	1.61
D4	15	17.97	8.22	2.12
Difference	15	-11.43	4.70	1.21

95% CI for mean difference: (-14.03, -8.82)
T-Test of mean difference = 0 (vs not = 0): T-Value = -9.41 P-Value = 0.000
SIGNIFICANT

P< 0.001

Field cecid trajectory radii on Pure Cultures.

Paired T-Test and Confidence Interval

Paired T for Vc pure - Hf pure

	N	Mean	StDev	SE Mean
Vc pure	22	8.059	4.442	0.947
Hf pure	22	5.159	2.122	0.452
Difference	22	2.900	2.776	0.592

95% CI for mean difference: (1.669, 4.131)
T-Test of mean difference = 0 (vs not = 0): T-Value = 4.90 P-Value = 0.000
SIGNIFICANT

P< 0.001

Paired T-Test and Confidence Interval

Paired T for Ln Vc pure - Ln Hf pure

	N	Mean	StDev	SE Mean
Ln Vc pu	22	1.949	0.542	0.115
Ln Hf pu	22	1.546	0.467	0.100
Difference	22	0.4035	0.1743	0.0372

95% CI for mean difference: (0.3262, 0.4807)
T-Test of mean difference = 0 (vs not = 0): T-Value = 10.86 P-Value = 0.000
SIGNIFICANT

P< 0.001

Appendix 8: P.S.P. area statistics - acetate sheet method (Minitab for windows).
INTERACTIVE FUNGAL ZONE AREAS (cm^2) – Acetate cut and weigh method.

Bi-culture V.commedens and H.fuscum, Formation of Pseudo-Sclerotial Plate:

Regression CONTROL

Regressions against time on x axis;

The regression equation is
y = - 4.13 + 3.28 x

Predictor	Coef	StDev	T	P
Constant	-4.130	4.974	-0.83	0.420
x	3.2760	0.7003	4.68	0.000

S = 8.775 R-Sq = 61.0% R-Sq(adj) = 58.2%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	1685.0	1685.0	21.88	0.000
Residual Error	14	1078.0	77.0		
Total	15	2763.0			

P< 0.001

SIGNIFICANT

Macro is running ... please wait

Regression WITH CECIDS

The regression equation is
y = - 3.95 + 2.00 x

Predictor	Coef	StDev	T	P
Constant	-3.951	2.668	-1.48	0.161
x	2.0004	0.2224	8.99	0.000

S = 5.396 R-Sq = 85.2% R-Sq(adj) = 84.2%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	2355.3	2355.3	80.89	0.000
Residual Error	14	407.7	29.1		
Total	15	2763.0			

P< 0.001

SIGNIFICANT

BICULUTRE V.commedens and H.fuscum
AMALGAMATED DATA TO TEST DIFFERENCE IN RATES OF PRODUCTION OF PSP.

Paired T-Test and Confidence Interval

Paired T for Control - Cecids

	N	Mean	StDev	SE Mean
Control	16	6.37	3.24	0.81
Cecids	16	10.35	6.26	1.57
Difference	16	-3.975	3.657	0.914

95% CI for mean difference: (-5.924, -2.026)
T-Test of mean difference = 0 (vs not = 0): T-Value = -4.35 P-Value = 0.001

SIGNIFICANT P< 0.005

Paired T-Test and Confidence Interval

BICULTURE; amalgamated data on rate of production of melanised area in petri-dish with and without cecids.

Paired T for No Insects - insects

	N	Mean	StDev	SE Mean
No Insec	16	10.46	6.93	1.73
insects	16	7.96	3.87	0.97
Difference	16	2.51	5.99	1.50

95% CI for mean difference: (-0.69, 5.70)
T-Test of mean difference = 0 (vs not = 0): T-Value = 1.67 P-Value = 0.115

NOT SIGNIFICANT P>0.05

Area analysis is by acetate sheet (experiment 2)

Paired T-Test and Confidence Interval

```
Paired T for PSP CON - PSP +C

                N      Mean     StDev   SE Mean
PSP CON        10      3.75      0.66      0.21
PSP +C         10      8.13      5.83      1.84
Difference     10     -4.38      5.92      1.87

95% CI for mean difference: (-8.61, -0.15)
T-Test of mean difference = 0 (vs not = 0): T-Value = -2.34  P-Value = 0.044
```

SIGNIFICANT

P< 0.05

Paired T-Test and Confidence Interval

```
Paired T for Vc CON - Vc +C

                N      Mean     StDev   SE Mean
Vc CON         10     18.96      1.28      0.40
Vc +C          10     18.35      4.92      1.55
Difference     10      0.60      5.75      1.82

95% CI for mean difference: (-3.51, 4.71)
T-Test of mean difference = 0 (vs not = 0): T-Value = 0.33  P-Value = 0.747
```

NOT SIGNIFICANT

P>0.05

Paired T-Test and Confidence Interval

```
Paired T for Hf Mel +C - Hf Mel CON

                N      Mean     StDev   SE Mean
Hf Mel +       10     20.40      7.25      2.29
Hf Mel C       10     16.81      3.29      1.04
Difference     10      3.59      7.76      2.45

95% CI for mean difference: (-1.96, 9.14)
T-Test of mean difference = 0 (vs not = 0): T-Value = 1.46  P-Value = 0.177
```

NOT SIGNIFICANT

P>0.05

Paired T-Test and Confidence Interval

```
Paired T for Hf unpig + C - Hf unpig CON

                N      Mean     StDev   SE Mean
Hf unpig       10      0.62      0.58      0.18
Hf unpig       10     17.61      3.69      1.17
Difference     10    -16.99      3.68      1.16

95% CI for mean difference: (-19.62, -14.36)
T-Test of mean difference = 0 (vs not = 0): T-Value = -14.62  P-Value = 0.000
```

SIGNIFICANT

P< 0.001

Mann-Whitney Confidence Interval and Test

```
PSP raw     N =   9     Median =      3041.0
PSP CON     N =   9     Median =       668.0
Point estimate for ETA1-ETA2 is      2345.2
95.8 Percent CI for ETA1-ETA2 is (-128.7,2940.9)
W = 99.0
Test of ETA1 = ETA2  vs  ETA1 not = ETA2 is significant at 0.2510

Cannot reject at alpha = 0.05
```

P>0.05

NOT SIGNIFICANT

Mann-Whitney Confidence Interval and Test

```
Hf raw      N =   9     Median =       13297
Hf CON      N =   9     Median =       14747
Point estimate for ETA1-ETA2 is       -1495
95.8 Percent CI for ETA1-ETA2 is (-3262,-622)
W = 54.0
Test of ETA1 = ETA2  vs  ETA1 not = ETA2 is significant at 0.0062
The test is significant at 0.0062 (adjusted for ties)
```

P< 0.05

SIGNIFICANT

Mann-Whitney Confidence Interval and Test

```
Vc raw      N =   9     Median =      4475.0
Vc CON      N =   9     Median =      4559.0
Point estimate for ETA1-ETA2 is       -235.0
95.8 Percent CI for ETA1-ETA2 is (-1257.4,1189.0)
W = 81.0
Test of ETA1 = ETA2  vs  ETA1 not = ETA2 is significant at 0.7239

Cannot reject at alpha = 0.05
```

P>0.05

NOT SIGNIFICANT

Appendix 9: Combined culture area statistics
INTERACTIVE FUNGAL ZONE AREAS (cm^2) –image analysis (Minitab for windows).

COMBINATION CULTURES WITH MEMBRANE (top view)

Mann-Whitney Confidence Interval and Test (n=9)

```
PSP raw    N =   9     Median =      1547.0
PSP CON    N =   9     Median =       686.5
Point estimate for ETA1-ETA2 is       801.2
95.8 Percent CI for ETA1-ETA2 is (399.9,1233.0)
W = 126.0
Test of ETA1 = ETA2  vs  ETA1 not = ETA2 is significant at 0.0004
SIGNIFICANT
```

P< 0.001

COMBINED CULUTRE AREA IMAGE ANALYSIS (AMALGAMATED RESULTS n=37)

Paired T-Test and Confidence Interval

Paired T for PSP + c - PSP con (n=37)

	N	Mean	StDev	SE Mean
PSP + c	37	2802	2679	440
PSP con	37	563	216	36
Difference	37	2240	2765	455

95% CI for mean difference: (1318, 3162)
T-Test of mean difference = 0 (vs not = 0): T-Value = 4.93 P-Value = 0.000

SIGNIFICANT

P< 0.001

Paired T-Test and Confidence Interval

Paired T for Hf + c - Hf con

	N	Mean	StDev	SE Mean
Hf + c	37	13264	3100	510
Hf con	37	14960	1418	233
Difference	37	-1696	2460	404

95% CI for mean difference: (-2516, -876)
T-Test of mean difference = 0 (vs not = 0): T-Value = -4.19 P-Value = 0.000
SIGNIFICANT

P< 0.001

Paired T-Test and Confidence Interval (n=37)

Paired T for Vc + c - Vc con

	N	Mean	StDev	SE Mean
Vc + c	37	3926	1086	179
Vc con	37	4616	1515	249
Difference	37	-690	1358	223

95% CI for mean difference: (-1143, -237)
T-Test of mean difference = 0 (vs not = 0): T-Value = -3.09 P-Value = 0.004
SIGNIFICANT

P< 0.001

ASTERISKS SHOW SIGNIFICANTLY DIFFERENT AREAS: P< 0.005
PSP = pseudo sclerotial plate
Vc = *Vuilleminia commedens*
Hf = *Hypoxylon fuscum*

Appendix 10: Fishers' Exact Test for combined culture description frequency data (SPSS for windows).

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
PSPLIFT * PSPCRACK	27	100.0%	0	.0%	27	100.0%

PSPLIFT * PSPCRACK Crosstabulation

CROSSTABULATION
PSP LIFT vs PSP CRACKED

Count

		PSPCRACK		
		.00	1.00	Total
PSPLIFT	.00	17		17
	1.00	2	8	10
Total		19	8	27

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	19.326[b]	1	.000		
Continuity Correction[a]	15.680	1	.000		
Likelihood Ratio	22.807	1	.000		
Fisher's Exact Test				.000	.000
Linear-by-Linear Association	18.611	1	.000		
N of Valid Cases	27				

P< 0.001

a. Computed only for a 2x2 table

b. 1 cells (25.0%) have expected count less than 5. The minimum expected count is 2.96.

Directional Measures

			Value	Asymp. Std. Error[a]
Nominal by Nominal	Lambda	Symmetric	.778	.156
		PSPLIFT Dependent	.800	.126
		PSPCRACK Dependent	.750	.198
	Goodman and Kruskal tau	PSPLIFT Dependent	.716	.152
		PSPCRACK Dependent	.716	.161
	Uncertainty Coefficient	Symmetric	.667	.149
		PSPLIFT Dependent	.641	.161
		PSPCRACK Dependent	.695	.135
Nominal by Interval	Eta	PSPLIFT Dependent	.846	
		PSPCRACK Dependent	.846	

Crosstabs

CROSSTABULATION

VC INVASION vs PSP RIDGE

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
VCINVAS * PSPRIDGE	27	100.0%	0	.0%	27	100.0%

VCINVAS * PSPRIDGE Crosstabulation

Count

		PSPRIDGE		
		.00	1.00	Total
VCINVAS	.00	4	14	18
	1.00	7	2	9
Total		11	16	27

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	7.670[b]	1	.006		
Continuity Correction[a]	5.542	1	.019		
Likelihood Ratio	7.895	1	.005		
Fisher's Exact Test				.011	.009
Linear-by-Linear Association	7.386	1	.007		
N of Valid Cases	27				

P< 0.005

a. Computed only for a 2x2 table

b. 1 cells (25.0%) have expected count less than 5. The minimum expected count is 3.67.

Directional Measures

			Value	Asymp. Std. Error[a]
Nominal by Nominal	Lambda	Symmetric	.400	.234
		VCINVAS Dependent	.333	.301
		PSPRIDGE Dependent	.455	.201
	Goodman and Kruskal tau	VCINVAS Dependent	.284	.177
		PSPRIDGE Dependent	.284	.174
	Uncertainty Coefficient	Symmetric	.223	.145
		VCINVAS Dependent	.230	.149
		PSPRIDGE Dependent	.216	.142
Nominal by Interval	Eta	VCINVAS Dependent	.533	
		PSPRIDGE Dependent	.533	

CROSSTABULATION

Crosstabs

PSP Diffuse vs PSP Sharp

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
PSPDIFFU * PSPSHARP	27	100.0%	0	.0%	27	100.0%

PSPDIFFU * PSPSHARP Crosstabulation

Count

		PSPSHARP		
		.00	1.00	Total
PSPDIFFU	.00		4	4
	1.00	23		23
Total		23	4	27

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	27.000[b]	1	.000		
Continuity Correction[a]	19.657	1	.000		
Likelihood Ratio	22.652	1	.000		
Fisher's Exact Test				.000	.000
Linear-by-Linear Association	26.000	1	.000		
N of Valid Cases	27				

P< 0.001

a. Computed only for a 2x2 table

b. 3 cells (75.0%) have expected count less than 5. The minimum expected count is .59.

Directional Measures

			Value	Asymp. Std. Error[a]
Nominal by Nominal	Lambda	Symmetric	1.000	.000
		PSPDIFFU Dependent	1.000	.000
		PSPSHARP Dependent	1.000	.000
	Goodman and Kruskal tau	PSPDIFFU Dependent	1.000	.000
		PSPSHARP Dependent	1.000	.000
	Uncertainty Coefficient	Symmetric	1.000	.000
		PSPDIFFU Dependent	1.000	.000
		PSPSHARP Dependent	1.000	.000
Nominal by Interval	Eta	PSPDIFFU Dependent	1.000	
		PSPSHARP Dependent	1.000	

CROSSTABULATION

PSP LIFT vs SECONDARY FUNGAL GROWTH

Crosstabs

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
PSPLIFT * SECONDGR	27	100.0%	0	.0%	27	100.0%

PSPLIFT * SECONDGR Crosstabulation

Count

		SECONDGR		
		.00	1.00	Total
PSPLIFT	.00	4	13	17
	1.00	8	2	10
Total		12	15	27

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	8.132[b]	1	.004		
Continuity Correction[a]	6.006	1	.014		
Likelihood Ratio	8.538	1	.003		
Fisher's Exact Test				.007	.007
Linear-by-Linear Association	7.831	1	.005		
N of Valid Cases	27				

P< 0.05

a. Computed only for a 2x2 table

b. 1 cells (25.0%) have expected count less than 5. The minimum expected count is 4.44.

Directional Measures

			Value	Asymp. Std. Error[a]
Nominal by Nominal	Lambda	Symmetric	.455	.214
		PSPLIFT Dependent	.400	.268
		SECONDGR Dependent	.500	.186
	Goodman and Kruskal tau	PSPLIFT Dependent	.301	.176
		SECONDGR Dependent	.301	.175
	Uncertainty Coefficient	Symmetric	.235	.146
		PSPLIFT Dependent	.240	.148
		SECONDGR Dependent	.230	.144
Nominal by Interval	Eta	PSPLIFT Dependent	.549	
		SECONDGR Dependent	.549	

CROSSTABULATION

PSP CRAKED vs SECONDARY FUNGAL GROWTH

Crosstabs

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
PSPCRACK * SECONDGR	27	100.0%	0	.0%	27	100.0%

PSPCRACK * SECONDGR Crosstabulation

Count

		SECONDGR		
		.00	1.00	Total
PSPCRACK	.00	6	13	19
	1.00	6	2	8
Total		12	15	27

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	4.299[b]	1	.038		
Continuity Correction[a]	2.720	1	.099		
Likelihood Ratio	4.400	1	.036		
Fisher's Exact Test				.087	.049
Linear-by-Linear Association	4.139	1	.042		
N of Valid Cases	27				

P< 0.05

a. Computed only for a 2x2 table

b. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.56.

Directional Measures

			Value	Asymp. Std. Error[a]
Nominal by Nominal	Lambda	Symmetric	.200	.115
		PSPCRACK Dependent	.000	.000
		SECONDGR Dependent	.333	.192
	Goodman and Kruskal tau	PSPCRACK Dependent	.159	.139
		SECONDGR Dependent	.159	.137
	Uncertainty Coefficient	Symmetric	.126	.113
		PSPCRACK Dependent	.134	.120
		SECONDGR Dependent	.119	.108
Nominal by Interval	Eta	PSPCRACK Dependent	.399	
		SECONDGR Dependent	.399	

APPENDIX 11

Table 7.1 *V.commedens* treated wood slice initial condition correlations.

asterisks=significant & strong (common to both tables).
rY=inter medullary ray distance.
b'rk thik'ns = bark thickness
hardn's = bark hardness.

Key: 1
bold = slope > 0.1

Correlations
INITIAL / INITIAL

Strength	VC rY	VC diam	VC age	VC hardn's	VC b'rk thik'ns	VC bark area
VC rY	1	0.008	0.021	0.099	0.001	0.005
VC diam		1	0.087	0.008	0.059	0.029
VC age			1	**0.143**	**0.208**	0.005
VC hardn's				1	0.096	0.04
VC b'rk thik'ns					1	**0.136**
VC bark area						1

1

Key: 2
bold = P <0.05
*and **bold** = P < 0.05 + in other table

Correlations
INITIAL / INITIAL

P.nos	VC rY	VC diam	VC age	VC hardn's	VC b'rk thik'ns	VC bark area
VC rY	Zero	0.49	0.25	**0.013**	0.8	0.58
VC diam		Zero	**0.02**	0.5	0.05	0.18
VC age			Zero	*0.002	*0.0001	0.6
VC hardn's				Zero	**0.01**	0.12
VC b'rk thik'ns					Zero	*0.003
VC bark area						Zero

2

Key: INITIAL = initial wood-slice conditions measured, EMERGENT = final conditions measured after decomposition VC = Vuilleminia commedens, Hf = Hypoxylon fuscum, Combi = VC + Hf. rY = distance between medullary rays (mm), diam = diameter (cm), hardn's = hardness (N/cm2), b;rk thikn's = bark thickness (mm),

Table 7.2 *H.fuscum* treated wood slice initial condition correlations.

asterisks=significant & strong (common to both tables).rY=inter medullary ray distance. b'rk thik'ns = bark thickness, hardn's = bark hardness

Correlation INITIAL / INITIAL

strength	Hf rY	Hf diam	Hf age	Hf hardn's	Hf b'rk thik'ns	Hf bark area
Hf rY	1	-0.13	0.103	**-0.448**	0.19	**0.344**
Hf diam		1	**0.464**	**0.254**	**0.291**	0.079
Hf age			1	**-0.22**	**0.61**	**0.195**
Hf hardn's				1	**-0.361**	**-0.21**
Hf b'rk thik'ns					1	**0.152**
Hf bark area						1

Key:
bold = slope > 0.1

Correlation INITIAL / INITIAL

P.nos	Hf rY	Hf diam	Hf age	Hf hardn's	Hf b'rk thik'ns	Hf bark area
Hf rY	Zero	0.313	0.425	✗ **0.0001**	0.138	✗ **0.006**
Hf diam		Zero	✗ **0.0001**	✗ **0.046**	✗ **0.022**	0.544
Hf age			Zero	0.086	✗ **0.0001**	0.128
Hf hardn's				Zero	✗ **0.004**	0.101
Hf b'rk thik'ns					Zero	0.238
Hf bark area						Zero

Key:
bold = P <0.05
*and **bold** = P < 0.05 + slope > 0.1

Key: INITIAL = initial wood-slice conditions measured, EMERGENT = final conditions measured after decomposition VC = Vuilleminia commedens, Hf = Hypoxylon fuscum, Combi = VC + Hf. rY = distance between medullary rays (mm), diam = diameter (cm), hardn's = harndness (N/cm2), b;rk thikn's = bark thickness (mm),

Table 7.3 Combined *H. fuscum*+ *V. commedens* treated wood slice initial condition correlations.

asterisks = significant & strong (common to both tables). rY = inter medullary ray distance, b'rk thik'ns = bark thickness, hardn's = bark hardness.

Key:
bold = slope > 0.1

Correlation INITIAL / INITIAL

strength	Combi rY	Combi diam	Combi age	Combi hardn's	Combi b'rk thik'ns	Combi bark area
Combi rY	1	0.07	0.005	-0.071	0.019	**0.114**
Combi diam		1	**0.273**	**0.394**	**0.387**	-0.036
Combi age			1	-0.058	**0.628**	**0.188**
Combi hardn's				1	**-0.205**	**-0.398**
Combi b'rk thik'ns					1	**0.303**
Combi bark area						1

Key:
bold = P <0.05
*and **bold** = P < 0.05 + slope > 0.1

Correlation INITIAL / INITIAL

P. nos	Combi rY	Combi diam	Combi age	Combi hardn's	Combi b'rk thik'ns	Combi bark area
Combi rY	zero	0.576	0.971	0.574	0.881	0.365
Combi diam		zero	* **0.026**	* **0.001**	* **0.001**	0.775
Combi age			zero	0.645	* **0.0001**	0.131
Combi hardn's				zero	0.098	* **0.001**
Combi b'rk thik'ns					zero	* **0.013**
Combi bark area						zero

Key: INITIAL = initial wood-slice conditions measured, EMERGENT = final conditions measured after decomposition VC = Vuilleminia commedens, Hf = Hypoxylon fuscum, Combi = VC + Hf. rY = distance between medullary rays (mm), diam = diameter (cm), hardn's = harndness (N/cm2), b;rk thikn's = bark thickness (mm),

Table 7.4 Control (latent fungal) treated wood slice initial condition correlations.

asterisks = significant & strong (common to both tables). rY = inter medullary ray distance, b'rk thik'ns = bark thickness, hardn's = bark hardness.

Key:
bold = slope > 0.1

Correlation INITIAL / INITIAL							
Strength	Con rY	Con diam	Con age	Con hardn's	Con b'rk thik'ns	Con bark are
Con rY	1	0.196	-0.097	-0.077	**-0.29**	-0.072
Con diam		1	**0.192**	**0.379**	**0.124**	0.002
Con age			1	0.068	**0.404**	**0.17**
Con hardn's				1	**0.21**	**-0.315**
Con b'rk thik'ns					1	**0.129**
Con bark area						1
Con EXP						

Key:
bold = P <0.05
*and **bold** = P < 0.05 + slope > 0.1

Correlation INITIAL / INITIAL						
P. nos	Con rY	Con diam	Con age	Con hardn's	Con b'rk thik'ns	Con bark are
Con rY	zero	0.213	0.543	0.627	0.062	0.649
Con diam		zero	0.224	**0.013** *	0.434	0.992
Con age			zero	0.669	**0.008** *	0.28
Con hardn's				zero	0.181	**0.042** *
Con b'rk thik'ns					zero	0.414
Con bark area						zero
Con EXP						

Key: INITIAL = initial wood-slice conditions measured, EMERGENT = final conditions measured after decomposition VC = Vuilleminia commedens, Hf = Hypoxylon fuscum, Combi = VC + Hf. rY = distance between medullary rays (mm), diam = diameter (cm), hardn's = hamdness (N/cm2), b;rk thikn's = bark thickness (mm),

Table 7.5 Amalgamated wood slice data initial condition correlations.

asterisks = significant & strong (common to both tables). rY = inter medullary ray distance, b'rk thik'ns = bark thickness, hardn's = bark hardness.

Key: **bold** = slope > 0.1

Correlation ALL INITIAL / INITIAL Strength	diameter before	bark thickness	All rY	All age	bark hardness befor	wood thickness	bark area
diameter before decompositi	1	0.06	0.001	**0.118**	0.089	0.06	0.004
bark thickness before decomposition		1	0	**0.348**	0.038	**0.33**	0.032
All rY			1	0	0.046	0	0.044
All age				1	0.016	**0.34**	0.035
bark hardness before decomposition					1	0.038	0.067
wood thickness						1	0.031
bark area							1

Key: **bold** = P <0.05 *and **bold** = P < 0.05 + slope > 0.1

Correlation ALL INITIAL / INITIAL P. nos	diameter before	bark thickness	All rY	All age	bark hardness befor	wood thickness	bark area
diameter before decompositi	Zero	**0.001**	0.73	* **0.0001**	**0.001**	**0.001**	0.4
bark thickness before decomposition		Zero	0.83	* **0.0001**	**0.01**	* **0.001**	**0.02**
All rY			Zero	0.82	**0.005**	0.8	**0.006**
All age				Zero	0.09	* **0.0001**	**0.015**
bark hardness before decomposition					Zero	**0.01**	**0.001**
bark thickness before decomposition						Zero	**0.02**
bark area							Zero

Key: INITIAL = initial wood-slice conditions measured, EMERGENT = final conditions measured after decomposition VC = Vuilleminia commedens, Hf = Hypoxylon fuscum, Combi = VC + Hf. rY = distance between medullary rays (mm), diam = diameter (cm), hardn's = harndness (N/cm2), b;rk thikn's = bark thickness (mm), ALL = COMPILED DATA FOR ALL TREATMENTS

Table 7.6 *V. commedens* treated wood slice emergent condition correlations.

asterisks = significant and strong (common to both tables). b'rk thik'ns = bark thickness, hardn's = bark hardness, ht lif'bark = height of lifted bark, no D columns = number of fungal decay columns or genets / wood slice, length PSP = length of PSP in contact with bark, full pen hardn's = hardness of underlying sap wood, cavities = number of cavities in sub-cortical zone.

Correlations
EMERGENT / EMERGENT

Strength	VC diam	VC b'rk thik'ns	Vcavities	VClength lif'brk	VCht lft brk	VCno D.colums	VClengh PSP	Vccecids	VChardn's	VCfull pen hardn's	VCno. colours	VCpignmentation
VC diam	1	0.088	0.007	0.005	0.001	0.048	0.036	0.017	0.015	0.022	0.01	0.064
VC b'rk thik'ns		1	0.006	0.001	0.015	0.001	0	0.003	0.001	0	0	0
Vcavities			1	0.011	**0.146**	0.028	0	0.084	0.016	0.027	0.041	0.001
VClength lif'brk				1	0.008	**0.219**	0.001	0.002	0	0.025	0.017	0.001
VCht lft brk					1	0.033	0.006	0.004	0.054	0.046	0.009	0.073
VCno D.colums						1	0.033	0.035	0.02	0.02	0.011	0.001
VClengh PSP							1	0.091	0.022	0.007	0.04	0.058
Vccecids								1	0.065	0.083	0.036	0.082
VChardn's									1	**0.194**	0.001	0.002
VCfull pen hardn's										1	0.001	0
VCno. colours											1	0.059
VCpignmentation												1

Key:
bold = slope > 0.1

REGRESSIONS
EMERGENT / EMERGENT

P. nos	VC diam	VC b'rk thik'ns	Vcavities	VClength lif'brk	VCht lft brk	VCno D.columns	VClengh PSP	Vccecids	VChardn's	VCfull pen hardn's	VCno. colours	VCpignmentation
VC diam	Zero	**0.02**	0.53	0.6	0.8	0.09	0.15	0.3	0.36	0.3	0.4	0.049
VC b'rk thik'ns		Zero	0.54	0.78	0.3	0.8	0.9	0.7	0.8	0.9	0.8	0.9
Vcavities			Zero	0.4	* **0.002**	0.19	0.9	**0.02**	0.3	0.2	0.11	0.8
VClength lif'brk				Zero	0.49	* **0.0001**	0.8	0.7	0.9	0.2	0.3	0.8
VCht lft brk					Zero	0.15	0.5	0.6	0.07	0.09	0.45	**0.03**
VCno D.colums						Zero	0.15	0.15	0.3	0.3	0.4	0.85
VClengh PSP							Zero	**0.017**	0.24	0.5	0.12	0.05
Vccecids								Zero	**0.04**	**0.02**	0.14	**0.02**
VChardn's									Zero	* **0.0001**	0.8	0.7
VCfull pen hardn's										Zero	0.8	0.9
VCno. colours											Zero	**0.05**
VCpignmentation												Zero

Key:
bold = P <0.05
*and **bold** = P < 0.05 + slope > 0.1

Key: INITIAL = initial wood-slice conditions measured, EMERGENT = final conditions measured after decomposition VC = Vuilleminia commedens, Hf = Hypoxylon fuscum, Combi = VC + Hf. rY = distance between medullary rays (mm), diam = diameter (cm), hardn's = harndness (N/cm2), b;rk thikn's = bark thickness (mm),

3 asterisks = significant and strong (common to both tables).

Table 7.7 *H. fuscum* treated wood slice emergent condition correlations.

APPENDIX 11

asterisks = significant and strong (common to both tables). b'rk thik'ns = bark thickness, hardn's = bark hardness, ht lif'bark = height of lifted bark, no D columns = number of fungal decay columns or genets / wood slice, length PSP = length of PSP in contact with bark, full pen hardn's = hardness of underlying sap wood, cavities = number of cavities in sub-cortical zone.

Key:
bold = slope > 0.1
bold = P <0.05
*and **bold** = P < 0.05 + slope > 0.1

Correlation EMERGENT / EMERGENT

strength	Hf diam	Hf b'rk thik'n	Hfcavities?	Hf length lif'l	Hfht lft brk	Hf no D.col	Hf lengh PSI	Hf lengh Hf	Hf cecids	Hf hardn's	Hf full pen h	Hf no. colou	Hf pignment
Hf diam	1	**0.245**	**0.328**	-0.027	**0.273**	**0.314**	**0.238**	**0.15**	**0.125**	**0.176**	**-0.221**	-0.049	**0.202**
Hf b'rk thik'ns		1	**0.235**	**0.187**	**0.288**	**0.174**	**0.142**	-0.04	**0.222**	**-0.292**	**-0.42**	-0.063	0.063
Hfcavities			1	0.092	0.089	0.064	0.029	**0.278**	0.2	**-0.205**	**-0.291**	-0.007	-0.101
Hf length lif'brk				1	**0.242**	**0.13**	**0.156**	**0.199**	-0.024	-0.075	-0.029	0.01	**0.29**
Hfht lft brk					1	0.028	0.061	0.015	0.111	**-0.211**	**-0.357**	**0.234**	**0.191**
Hf no D.colums						1	**0.847**	-0.052	**0.154**	-0.013	**-0.338**	**0.285**	**0.206**
Hf lengh PSP							1	-0.01	0.005	-0.051	**-0.289**	**0.167**	**0.208**
Hf lengh Hf								1	-0.038	**0.163**	0.027	-0.077	-0.052
Hf cecids									1	-0.211	**-0.287**	-0.01	**0.334**
Hf hardn's										1	**0.345**	**-0.271**	**-0.108**
Hf full pen hardn's											1	**-0.133**	-0.03
Hf no. colours												1	**0.301**
Hf pignmentation													1

Correlation EMERGENT / EMERGENT

p.nos	Hf diam	Hf b'rk thik'n	Hfcavities?	Hf length lif'l	Hfht lft brk	Hf no D.col	Hf lengh PSI	Hf lengh Hf	Hf cecids	Hf hardn's	Hf full pen h	Hf no. colou	Hf pignment
Hf diam	zero	0.057	**0.01**	0.837	**0.033**	**0.014**	0.064	0.249	0.335	0.174	0.087	0.773	0.23
Hf b'rk thik'ns		zero	0.068	0.15	**0.024**	0.181	0.276	0.759	0.086	**0.022**	**0.001**	0.712	0.709
Hfcavities			zero	0.478	0.49	0.62	0.825	**0.028**	0.12	0.109	**0.022**	0.969	0.551
Hf length lif'brk				zero	0.058	0.314	0.226	0.12	0.855	0.563	0.822	0.952	0.082
Hfht lft brk					zero	0.83	0.64	0.906	0.389	0.099	**0.004**	0.164	0.257
Hf no D.colums						zero	**0.0001**	0.691	0.231	0.919	**0.007**	0.087	0.221
Hf lengh PSP							zero	0.939	0.968	0.694	**0.023**	0.322	0.217
Hf lengh Hf								zero	0.768	0.206	0.836	0.65	0.759
Hf cecids									zero	0.1	**0.024**	0.954	**0.044**
Hf hardn's										zero	**0.006**	**0.015**	0.525
Hf full pen hardn's											zero	0.431	0.862
Hf no. colours												zero	0.07
Hf pignmentation													zero

16 asterisks = significant and strong (common to both tables).

Key: INITIAL = initial wood-slice conditions measured, EMERGENT = final conditions measured after decomposition VC = Vuilleminia commedens, Hf = Hypoxylon fuscum, Combi = VC + Hf. rY = distance between medullary rays (mm), diam = diameter (cm), hardn's = harndness (N/cm2), b;rk thikn's = bark thickness (mm),

Table 7.8 Combined *H. fuscum* + *V. commedens* treated wood slice emergent condition correlations.

asterisks = significant and strong (common to both tables). b'rk thik'ns = bark thickness, hardn's = bark hardness, ht lif'bark = height of lifted bark, no D columns = number of fungal decay columns or genets / wood slice, length PSP = length of PSP in contact with bark, full pen hardn's = hardness of underlying sap wood, cavities = number of cavities in sub-cortical zone.

Correlation EMERGENT / EMERGENT

Strength. Combi diam	Combi b'rk thik'ns	Combi cavities	Combi length lif'brk	Combi ht lft brk	Combi no D.colums	Combi lengh PSP	Combi lengh Hf	Combi cecids	Combi hardn's	Combi full pen hardn's	Combi no. col	Combi pignmentation
Combi diar 1	**-0.377**	0.074	0.085	0.09	0.044	**0.148**	-0.032	0.042	**0.245**	0.058	**0.16**	-0.169
Combi b'rk thik'ns	1	-0.014	-0.016	-0.057	0.004	0.036	-0.044	-0.018	-0.146	-0.013	**-0.255**	0.358
Combi cavities		1	**0.261**	**0.386**	**0.28**	-0.015	-0.082	0.088	-0.297	**-0.3**	-0.052	**0.464**
Combi length lif'brk			1	**0.254**	0.063	**0.282**	**0.235**	-0.031	0.031	-0.089	0.098	**0.26**
Combi ht lft brk				1	0.005	-0.019	-0.077	**0.362**	**0.142**	**-0.12**	**0.326**	**0.447**
Combi no D.colums					1	**0.174**	-0.014	**0.462**	-0.282	-0.061	**0.126**	**0.384**
Combi lengh PSP						1	-0.015	0.063	-0.025	0.047	**0.16**	**0.165**
Combi lengh Hf							1	-0.052	-0.023	-0.034	0.062	-0.032
Combi cecids								1	-0.2	-0.09	**0.329**	**0.342**
Combi hardn's									1	**0.267**	**0.241**	-0.163
Combi full pen hardn's										1	-0.049	-0.163
Combi no. colours											1	0.084
Combi pignmentation												1

bold = slope > 0.1

17 asterisks = significant and strong (common to both tables).

Correlation EMERGENT / EMERGENT

P. nos. Combi diam	Combi b'rk thik'ns	Combi cavities	Combi length lif'brk	Combi ht lft brk	Combi no D.colums	Combi lengh PSP	Combi lengh Hf	Combi cecids	Combi hardn's	Combi full pen hardn's	Combi no. col	Combi pignmentation
Combi diar zero	**0.002**	0.559	0.507	0.479	0.73	0.244	0.802	0.739	0.053	0.649	0.331	0.317
Combi b'rk thik'ns	zero	0.915	0.903	0.654	0.977	0.775	0.728	0.89	0.252	0.921	0.117	**0.03**
Combi cavities		zero	**0.038**	**0.002**	**0.025**	0.908	0.521	0.492	**0.018**	**0.016**	0.754	**0.004**
Combi length lif'brk			zero	**0.043**	0.619	**0.024**	0.062	0.807	0.81	0.486	0.552	0.12
Combi ht lft brk				zero	0.969	0.88	0.544	**0.003**	0.268	0.345	**0.043**	**0.006**
Combi no D.colums					zero	0.17	0.913	**0.0001**	**0.025**	0.632	0.446	**0.019**
Combi lengh PSP						zero	0.9	0.619	0.844	0.712	0.33	0.33
Combi lengh Hf							zero	0.684	0.856	0.79	0.706	0.85
Combi cecids								zero	0.117	0.481	**0.041**	**0.038**
Combi hardn's									zero	**0.034**	0.14	0.335
Combi full pen hardn's										zero	0.766	0.335
Combi no. colours											zero	0.619
Combi pignmentation												zero

Key:
bold = P <0.05
*and **bold** = P < 0.05 + slope > 0.1

Key: INITIAL = initial wood-slice conditions measured, EMERGENT = final conditions measured after decomposition VC = Vuilleminia commedens, Hf = Hypoxylon fuscum, Combi = VC + Hf. rY = distance between medullary rays (mm), diam = diameter (cm), hardn's = harndness (N/cm2), b;rk thikn's = bark thickness (mm),

Table 7.9 Control (latent fungal) treated wood slice emergent condition correlations.

asterisks = significant and strong (common to both tables). b'rk thik'ns = bark thickness, hardn's = bark hardness, ht lif'bark = height of lifted bark, no D columns = number of fungal decay columns or genets / wood slice, length PSP = length of PSP in contact with bark, full pen hardn's = hardness of underlying sap wood, cavities = number of cavities in sub-cortical zone.

Correlation EMERGENT / EMERGENT

Strength	Con diam	Con b'rk thik'ns	Con cavities	Con length lif'brk	Con ht lft brk	Con no D.colums	Con lengh PSP	Con lengh Hf	Con cecids	con hardn's	Con full pen hardn's	Con no. colours	Con pignmentation
Con diam	1	**-0.251**	0.008	**0.151**	**0.33**	**0.167**	-0.078	**-0.238**	-0.0001	0.175	-0.024	**-0.196**	**-0.127**
Con b'rk thik'ns		1	**0.259**	**-0.174**	**0.177**	0.039	**0.184**	**0.222**	**-0.181**	0.077	**-0.19**	**-0.103**	0.022
Con cavities			1	0.048	**0.554**	0.078	**-0.126**	**0.154**	-0.076	-0.073	-0.38	**-0.204**	**0.21**
Con length lif'brk				1	**0.277**	-0.039	-0.097	**-0.114**	-0.078	-0.137	0.007	-0.021	**-0.141**
Con ht lft brk					1	0.058	**-0.114**	-0.096	-0.093	**0.319**	**-0.345**	**-0.307**	**0.168**
Con no D.colums						1	**0.113**	0.037	**0.415**	-0.094	**-0.146**	**-0.244**	**0.307**
Con lengh PSP							1	0.01	-0.065	-0.022	-0.081	-0.0001	**0.16**
Con lengh Hf								1	-0.072	-0.103	0.028	-0.036	0.056
Con cecids									1	-0.089	**-0.14**	-0.078	**0.23**
con hardn's										1	**0.176**	**0.223**	**-0.196**
Con full pen hardn's											1	**0.447**	**-0.217**
Con no. colours												1	**-0.168**
Con pignmentation													1

Key:
bold = slope > 0.1

Correlation EMERGENT / EMERGENT

P.nos	Con diam	Con b'rk thik'ns	Con cavities	Con length lif'brk	Con ht lft brk	Con no D.colums	Con lengh PSP	Con lengh Hf	Con cecids	con hardn's	Con full pen hardn's	Con no. colours	Con pignmentation
Con diam	zero	0.118	0.962	0.352	* **0.037**	0.302	0.631	0.139	0.999	0.286	0.883	0.273	0.481
Con b'rk thik'ns		zero	0.107	0.282	0.276	0.81	0.255	0.168	0.263	0.643	0.246	0.567	0.902
Con cavities			zero	0.769	* **0.0001**	0.634	0.437	0.342	0.643	0.66	**0.017**	0.255	0.241
Con length lif'brk				zero	* **0.05**	0.812	0.552	0.484	0.631	0.406	0.967	0.906	0.434
Con ht lft brk					zero	0.72	0.484	0.554	0.568	0.048	* **0.032**	* **0.06**	0.351
Con no D.colums						zero	0.488	0.823	* **0.008**	0.569	0.376	0.172	0.082
Con lengh PSP							zero	0.951	0.692	0.894	0.622	1	0.374
Con lengh Hf								zero	0.658	0.532	0.865	0.841	0.759
Con cecids									zero	0.59	0.396	0.665	0.198
con hardn's										zero	0.285	0.212	0.273
Con full pen hardn's											zero	* **0.009**	0.224
Con no. colours												zero	0.35
Con pignmentation													zero

Key:
bold = P <0.05
*and **bold** = P < 0.05 + slope > 0.1

7 asterisks = strong and significant (common to both tables).

Key: INITIAL = initial wood-slice conditions measured, EMERGENT = final conditions measured after decomposition VC = Vuilleminia commedens, Hf = Hypoxylon fuscum, Combi = VC + Hf. rY = distance between medullary rays (mm), diam = diameter (cm), hardn's = harndness (N/cm2), b;rk thikn's = bark thickness (mm),

Table 7.10 Amalgamated wood slice data emergent condition correlations.

asterisks = significant and strong (common to both tables). b'rk thik'ns = bark thickness, hardn's = bark hardness, ht lif'bark = height of lifted bark, no D columns = number of fungal decay columns or genets / wood slice, length PSP = length of PSP in contact with bark, full pen hardn's = hardness of underlying sap wood, cavities = number of cavities in sub-cortical zone.

Correlations ALL EMERGENT / EMERGENT

Strength	diameter after	bark thickness after	All cavities	All length lif'brk	All ht lft brk	All no D.colums	All lengh PSP	All lengh Hf	All cecids	bark hardness after	wood hardness after	All no. colours	All pignmentation
diameter after decomposition	1	0.008	0.016	0.005	0.006	0.037	0.054	0.089	0.029	0.007	0.008	0	0.009
bark thickness after decomposition		1	0.045	0	0.01	0.002	0.003	0.003	0.024	0.009	0.026	0.015	0.042
All cavities			1	0.009	0.014	0.004	0.002	0.004	**0.106**	0.06	0.049	0.009	0.069
All length lif'brk				1	0.004	0.001	0.008	0.009	0.037	0.014	0.057	0.01	0.03
All ht lft brk					1	0.008	0.025	0.068	0.054	0.005	0.046	0.001	0.028
All no D.colums						1	**0.14**	**0.17**	**0.105**	0.014	0.069	0.023	0.091
All lengh PSP							1	**0.41**	0.009	0.017	0.004	0.012	0.001
All lengh Hf								1	**0.221**	0.007	0.023	0.037	0.011
All cecids									1	0.041	0.004	0.07	**0.11**
bark hardness after decomposition										1	**0.21**	0.001	0.047
wood hardness after decomposition											1	0.001	0.065
All no. colours												1	0.053
All pignmentation													1

Key:
bold = slope > 0.1

Correlations ALL EMERGENT / EMERGENT

P nos	diameter after	bark thickness after	All cavities	All length lif'brk	All ht lft brk	All no D.colums	All lengh PSP	All lengh Hf	All cecids	bark hardness after	wood hardness after	All no. colours	All pignmentation
diameter after decomposition	Zero	0.24	0.14	0.46	0.4	**0.017**	0.124	0.23	0.28	0.28	0.26	0.8	0.32
bark thickness after decomposition		Zero	**0.013**	0.96	0.27	0.53	0.7	0.82	0.32	0.23	**0.04**	0.21	**0.04**
All cavities			Zero	0.29	0.2	0.45	0.8	0.8	**0.04**	**0.004**	**0.009**	0.39	**0.02**
All length lif'brk				Zero	0.47	0.73	0.6	0.73	0.27	0.19	**0.008**	0.4	0.15
All ht lft brk					Zero	0.33	0.8	0.33	0.18	0.44	0.018	0.8	0.16
All no D.colums						Zero	**0.01**	0.08	0.03	0.13	**0.001**	0.12	**0.002**
All lengh PSP							Zero	**0.03**	0.73	0.39	0.68	0.6	0.85
All lengh Hf								Zero	0.2	0.74	0.55	0.53	0.73
All cecids									Zero	0.2	0.7	0.125	**0.05**
bark hardness after decomposition										Zero	**0.0001**	0.74	**0.02**
wood hardness after decomposition											Zero	0.8	**0.009**
All no. colours												Zero	**0.02**
All pignmentation													Zero

Key:
bold = P <0.05
*and **bold** = P < 0.05 + slope > 0.1

5 asterisks = strong and significant (common to both tables).

Key: INITIAL = initial wood-slice conditions measured, EMERGENT = final conditions measured after decomposition VC = Vuilleminia commedens, Hf = Hypoxylon fuscum, Combi = VC + Hf. rY = distance between medullary rays (mm), diam = diameter (cm), hardn's = hamdness (N/cm2), b;rk thikn's = bark thickness (mm),

Table 7.11 *V. commedens* treated wood slice initial to emergent transition correlations.

10 asterisks = strong and significant (common to both tables). rY= inter medullary ray distance.

Key:
bold = slope > 0.1

Correlations
EMERGENT / INITIAL

Strength	VC rY	VC diam	VC age	VC hardn's	VC b'rk thik'n	VC bark are	INITIAL = horizontal
VC diam	0.09	**0.96**	**0.31**	0.065	0.065	**-0.174**	
VC b'rk thik'ns	**0.24**	**0.27**	**0.31**	**-0.363**	**-0.365**	**0.302**	
Vcavities	**0.15**	0.081	-0.047	-0.0171	-0.171	**-0.115**	
VClength lif'brk	**-0.31**	0.066	**-0.17**	0.1	0.1	0.106	
VCht lft brk	**-0.16**	**0.118**	**-0.18**	**0.21**	**0.21**	**0.172**	
VCno D.colums	-0.097	**0.248**	0.021	**0.173**	**0.173**	0.019	
VClengh PSP	-0.059	**-0.189**	-0.045	**0.143**	**0.14**	**0.144**	
Vccecids	**0.247**	**-0.137**	0.09	-0.092	-0.092	0.004	
VChardn's	0.084	-0.012	**-0.153**	**0.171**	**0.171**	**-0.123**	
VCfull pen hardn's	0.015	0.009	**-0.227**	-0.073	-0.073	**0.18**	
VCno. colours	-0.085	**-0.293**	-0.078	**0.209**	-0.014	0.08	
VCpignmentation	-0.004	**-0.261**	0.012	-0.0001	-0.092	0.087	

EMERGENT = vertical

Key:
bold = P <0.05
*and **bold** = P < 0.05 + slope > 0.1

Correlations
EMERGENT / INITIAL

P.nos	VC rY	VC diam	VC age	VC hardn's	VC b'rk thik'n	VC bark are	INITIAL = horizontal
VC diam	0.49	**0.0001**	**0.015**	0.616	0.616	0.18	
VC b'rk thik'ns	0.061	**0.035**	**0.015**	**0.004**	**0.004**	**0.018**	
Vcavities	0.246	0.533	0.719	0.184	0.184	0.374	
VClength lif'brk	0.014	0.613	0.186	0.441	0.441	0.414	
VCht lft brk	0.21	0.362	0.167	0.101	0.1	0.182	
VCno D.colums	0.455	**0.05**	0.87	0.18	0.18	0.882	
VClengh PSP	0.649	0.141	0.728	0.27	0.268	0.264	
Vccecids	0.053	0.288	0.488	0.478	0.478	0.976	
VChardn's	0.517	0.925	0.234	0.184	0.184	0.342	
VCfull pen hardn's	0.91	0.948	0.075	0.572	0.572	0.162	
VCno. colours	0.512	**0.021**	0.54	0.102	0.915	0.539	
VCpignmentation	0.98	**0.04**	0.928	0.998	0.478	0.503	

EMERGENT = vertical

asterisks = significant and strong (common to both tables). rY = inter medullary ray distance, b'rk thik'ns = bark thickness, hardn's = bark hardness, ht lif'bark = height of lifted bark, no D columns = number of fungal decay columns or genets / wood slice, length PSP = length of PSP in contact with bark, full pen hardn's = hardness of underlying sap wood, cavities = number of cavities in sub-cortical zone.

Key: INITIAL = initial wood-slice conditions measured, EMERGENT = final conditions measured after decomposition VC = Vuilleminia commedens, Hf = Hypoxylon fuscum, Combi = VC + Hf. rY = distance between medullary rays (mm), diam = diameter (cm), hardn's = hardness (N/cm2), b;rk thikn's = bark thickness (mm),

Table 7.12 *H. fuscum* treated wood slice initial to emergent transition correlations.

asterisks = significant and strong (common to both tables). rY = inter medullary ray distance, b'rk thik'ns = bark thickness, hardn's = bark hardness, ht lif'bark = height of lifted bark, no D columns = number of fungal decay columns or genets / wood slice, length PSP = length of PSP in contact with bark, full pen hardn's = hardness of underlying sap wood, cavities = number of cavities in sub-cortical zone.

Correlation INITIAL / EMERGENT

strength	Hf diam	Hf b'rk thik'ns	Hfcavities	Hf length lif'brk	Hfht lft brk	Hf no D.colums	Hf lengh PSP	Hf lengh Hf	Hf cecids	Hf hardn's	Hf full pen hardn's	Hf no. colours	Hf pignmentation	EMERGEN
Hf rY	-0.087	-0.063	0.047	-0.032	0.018	-0.018	-0.023	**0.115**	-0.019	0.006	0.063	-0.021	-0.222	
Hf diam	**0.91**	**0.247**	**0.39**	-0.088	**0.276**	0.283	**0.233**	0.17	**0.135**	0.037	**-0.257**	-0.085	0.172	
Hf age	**0.443**	**0.609**	**0.393**	0.094	**0.144**	**0.106**	0.042	0.058	**0.148**	-0.034	**-0.223**	**-0.253**	**-0.261**	
Hf hardn's	**0.336**	**-0.311**	0.006	**-0.144**	-0.023	**0.194**	**0.109**	0.028	-0.089	**0.296**	0.081	**0.219**	0.088	
Hf b'rk thik'	**0.237**	**0.496**	**0.244**	**0.135**	**0.162**	**0.106**	0.076	-0.04	**0.181**	**-0.292**	**-0.42**	-0.063	-0.002	
Hf bark are	0.048	**0.18**	**0.156**	**0.135**	**0.122**	**0.1**	**0.116**	**0.116**	0.093	**-0.27**	0.07	0.084	0.005	

Key: **bold** = slope > 0.1

INITIAL = vertical

Correlation INITIAL / EMERGENT

p.nos	Hf diam	Hf b'rk thik'ns	Hfcavities	Hf length lif'brk	Hfht lft brk	Hf no D.colums	Hf lengh PSP	Hf lengh Hf	Hf cecids	Hf hardn's	Hf full pen hardn's	Hf no. colours	Hf pignmentation	EMERGEN
Hf rY	0.504	0.627	0.719	0.806	0.889	0.889	0.859	0.374	0.886	0.965	0.624	0.902	0.187	
Hf diam	**0.0001**	**0.05**	**0.002**	0.496	**0.03**	**0.026**	0.069	0.186	0.295	0.776	**0.044**	0.617	0.309	
Hf age	**0.0001**	**0.0001**	**0.002**	0.468	0.263	0.414	0.749	0.653	0.251	0.795	0.082	0.116	0.119	
Hf hardn's	0.008	**0.015**	0.964	0.265	0.856	0.131	0.399	0.803	0.493	**0.019**	0.531	0.192	0.603	
Hf b'rk thik'	0.066	**0.0001**	0.056	0.295	0.208	0.413	0.557	0.75	0.158	**0.022**	**0.001**	0.712	0.99	
Hf bark are	0.712	0.165	0.225	0.296	0.347	0.437	0.371	0.37	0.472	**0.034**	0.587	0.62	0.978	

Key: **bold** = P <0.05 *and **bold** = P < 0.05 + slope > 0.1

INITIAL = vertical

Key: INITIAL = initial wood-slice conditions measured, EMERGENT = final conditions measured after decomposition VC = Vuilleminia commedens, Hf = Hypoxylon fuscum, Combi = VC + Hf. rY = distance between medullary rays (mm), diam = diameter (cm), hardn's = harndness (N/cm2), b;rk thikn's = bark thickness (mm),

14 asterisks = strong and significant (common to both tables). rY = inter medullary ray distance.

Table 7.13 Combined *H. fuscum* + *V. commedens* wood slice treatment initial to emergent transition correlations.

asterisks = significant and strong (common to both tables). rY = inter medullary ray distance , b'rk thik'ns = bark thickness, hardn's = bark hardness, ht lif'bark = height of lifted bark, no D columns = number of fungal decay columns or genets / wood slice, length PSP = length of PSP in contact with bark, full pen hardn's = hardness of underlying sap wood, cavities = number of cavities in sub-cortical zone.

Key:
bold = slope > 0.1

, Correlation Analysis

Correlation INITIAL / EMERGENT

Strength.	Combi diam	Combi b'rk thik'ns	Combi cavities	Combi length	Combi ht lft brk	Combi no D.columns	Combi lengh PSP	Combi lengh Hf	Combi cecids	Combi hardn's	Combi full pen hardn's	Combi no. colours	Combi pignmentation
Combi rY	-0.005	**-0.116**	-0.08	0.027	**0.232**	-0.071	0.02	-0.092	-0.049	**0.181**	-0.089	0.094	0.064
Combi diam	**0.769**	0.06	**-0.159**	0.089	-0.021	-0.002	**0.156**	-0.038	0.009	**0.247**	-0.021	0.087	-0.065
Combi age	0.009	**0.226**	-0.107	-0.18	-0.007	-0.006	**-0.139**	-0.07	-0.01	0.007	**-0.202**	-0.073	-0.083
Combi hardn's	**0.363**	0.052	0.066	**0.17**	**0.122**	-0.038	**0.322**	-0.078	**0.174**	**0.17**	-0.003	**0.232**	0.078
Combi b'rk thik'ns	0.073	**0.286**	-0.072	**-0.219**	0.002	-0.021	**-0.219**	-0.082	0.075	0.041	**-0.121**	-0.056	0.046
Combi bark area	-0.073	0.041	0.001	-0.04	0.029	**-0.143**	**-0.229**	0.021	**-0.194**	-0.001	0.03	-0.007	0.049

INITIAL = vertical

Correlation

P. nos	Combi diam	Combi b'rk thik'ns	Combi cavities	Combi length	Combi ht lft brk	Combi no D.colums	Combi lengh PSP	Combi lengh Hf	Combi cecids?	Combi hardn's	Combi full pen hardn's	Combi no. colours	Combi pignmentation
Combi rY	0.966	0.36	0.532	0.83	0.066	0.577	0.876	0.47	0.701	0.155	0.483	0.568	0.707
Combi diam	**0.0001**	0.637	0.209	0.486	0.869	0.988	0.219	0.763	0.943	0.051	0.87	0.598	0.7
Combi age	0.944	0.072	0.4	0.155	0.956	0.962	0.273	0.584	0.937	0.959	0.109	0.658	0.626
Combi hardn's	**0.003**	0.684	0.607	0.18	0.339	0.763	**0.009**	0.539	0.168	0.183	0.98	0.156	0.647
Combi b'rk thik'ns	0.564	**0.022**	0.572	0.082	0.99	0.869	0.082	0.519	0.555	0.752	0.342	0.733	0.785
Combi bark area	0.566	0.75	0.994	0.756	0.822	0.259	**0.05**	0.868	0.125	0.995	0.815	0.966	0.775

INITIAL = vertical

Key:
bold = P <0.05
*and **bold** = P < 0.05 + slope > 0.1

5 asterisks = strong and significant (common to both tables). rY = inter medullary ray distance.

Key: *INITIAL = initial wood-slice conditions measured, EMERGENT = final conditions measured after decomposition VC = Vuilleminia commedens, Hf = Hypoxylon fuscum, Combi = VC + Hf. rY = distance between medullary rays (mm), diam = diameter (cm), hardn's = harndness (N/cm2), b;rk thikn's = bark thickness (mm),*

Table 7.14 Control (latent fungal) wood slice treatment initial to emergent transition correlations.

asterisks = significant and strong (common to both tables). rY = inter medullary ray distance, b'rk thik'ns = bark thickness, hardn's = bark hardness, ht lif'bark = height of lifted bark, no D columns = number of fungal decay columns or genets / wood slice, length PSP = length of PSP in contact with bark, full pen hardn's = hardness of underlying sap wood, cavities = number of cavities in sub-cortical zone.

Correlation INITIAL / EMERGENT

Strength	Con diam	Con b'rk thik'ns	Con cavities	Con length lif'brk	Con ht lft brk	Con no D.colums	Con lengh PSP	Con lengh Hf	Con cecids	con hardn's	Con full pen hardn's	Con no. colours	Con pignmentation
Con rY	**0.217**	**-0.319**	0.026	-0.095	**0.113**	**-0.14**	**0.155**	**-0.205**	-0.028	**-0.133**	**-0.188**	**-0.463**	0.094
Con diam	**0.901**	**-0.128**	-0.015	0.091	**0.338**	**0.123**	-0.056	**-0.335**	-0.047	**0.222**	-0.022	**-0.188**	**-0.135**
Con age	**0.112**	**0.168**	-0.073	-0.049	**0.165**	**0.2**	-0.097	**-0.14**	-0.063	0.023	-0.085	-0.061	0.053
Con hardn'	**0.283**	**-0.133**	0.046	0.035	0.008	**0.212**	**-0.21**	**0.257**	**0.126**	0.001	**0.124**	-0.103	**0.16**
Con b'rk th	0.084	**0.129**	-0.076	0.037	0.039	**0.104**	0.036	**0.116**	-0.166	0.077	-0.026	**0.112**	**0.163**
Con bark a	**-0.139**	**0.319**	**-0.166**	-0.094	**-0.155**	-0.054	**0.155**	**-0.176**	**-0.111**	**-0.194**	-0.22	-0.058	0.093
Con EXP													

INITIAL = vertical

Key:
bold = slope > 0.1

Correlation INITIAL / EMERGENT

P.nos	Con diam	Con b'rk thik'ns	Con cavities	Con length lif'brk	Con ht lft brk	Con no D.colums	Con lengh PSP	Con lengh Hf	Con cecids	con hardn's	Con full pen hardn's	Con no. colours	Con pignmentation
Con rY	0.178	* **0.045**	0.871	0.561	0.487	0.387	0.34	0.204	0.864	0.42	0.252	* **0.007**	0.602
Con diam	* **0.0001**	0.432	0.929	0.578	* **0.033**	0.45	0.731	* **0.035**	0.775	0.175	0.895	0.295	0.452
Con age	0.49	0.301	0.652	0.764	0.309	0.216	0.553	0.389	0.7	0.89	0.607	0.734	0.77
Con hardn'	* **0.05**	0.488	0.777	0.828	0.961	0.19	0.194	0.109	0.438	0.994	0.45	0.568	0.375
Con b'rk th	0.607	0.426	0.641	0.823	0.813	0.525	0.827	0.477	0.305	0.643	0.873	0.535	0.364
Con bark a	0.391	* **0.045**	0.307	0.565	0.339	0.741	0.341	0.277	0.493	0.236	0.178	0.75	0.609
Con EXP													

INITIAL = vertical

Key:
bold = P <0.05
*and **bold** = P < 0.05 + slope > 0.1

7 asterisks = strong and significant (common to both tables). rY = inter medullary ray distance.

Key: INITIAL = initial wood-slice conditions measured, EMERGENT = final conditions measured after decomposition VC = Vuilleminia commedens, Hf = Hypoxylon fuscum, Combi = VC + Hf. rY = distance between medullary rays (mm), diam = diameter (cm), hardn's = harndness (N/cm2), b;rk thikn's = bark thickness (mm),

Table 7.15 Amalgamated wood slice data initial to emergent transition correlations.

6 asterisks = strong and significant (common to both tables). rY = inter medullary ray distance.

asterisks = significant and strong (common to both tables). rY = inter medullary ray distance, b'rk thik'ns = bark thickness, hardn's = bark hardness, ht lif'bark = height of lifted bark, no D columns = number of fungal decay columns or genets / wood slice, length PSP = length of PSP in contact with bark, full pen hardn's = hardness of underlying sap wood, cavities = number of cavities in sub-cortical zone.

rY = inter medullary ray distance.

Correllations ALL INITIAL (top) / EMERGENT (side)

Strength	diameter before	bark thickness before	All rY	All age	bark hardness before	wood thickness	bark area
diameter after decomposition	**0.95**	0.06	0.002	0.087	**0.104**	0.033	0.002
bark thickness after decomposition	0.018	**0.333**	0.011	**0.138**	0.004	**0.333**	0.029
All cavities?	0.018	0.018	0	0.007	0.002	0.018	0.002
All length lif'brk	0.005	0	0.003	0.006	0.012	0	0
All ht lft brk	0.003	0.009	0.003	0.003	0.001	0.009	0
All no D.colums	0.028	0.003	0	0.003	0.017	0.003	0
All lengh PSP	0.044	0.009	0.017	0.012	0.015	0.009	0.035
All lengh Hf	0.091	0.013	**0.209**	0.001	0.012	0.013	0.029
All cecids?	0.017	0.094	0.027	0.05	0.002	0.094	0.011
bark hardness after decomposition	0.01	0.001	0.001	0.002	0.031	0.001	0.019
wood hardness after decomposition	0.008	0	0.013	0.009	0	0	0
All no. colours	0	0.001	0.01	0.007	0.021	0.001	0.005
All pignmentation	0.012	0	0	0.016	0.034	0	0.005

Key:
bold = slope > 0.1

Correllations ALL INITIAL (top) / EMERGENT (side)

P.nos	diameter before	bark thickness before	All rY	All age	bark hardness before	wood thickness	bark area
diameter after decomposition	✱ **0.0001**	**0.001**	0.54	**0.001**	✱ **0.0001**	**0.01**	0.62
bark thickness after decomposition	0.089	✱ **0.0001**	0.19	✱ **0.0001**	0.44	✱ **0.0001**	**0.02**
All cavities?	0.116	0.12	0.9	0.32	0.57	0.12	0.63
All length lif'brk	0.43	0.996	0.56	0.416	0.23	0.996	0.82
All ht lft brk	0.543	0.31	0.58	0.56	0.79	0.3	0.78
All no D.colums	**0.03**	0.5	0.98	0.52	0.09	0.5	0.79
All lengh PSP	0.16	0.5	0.39	0.46	0.42	0.53	0.2
All lengh Hf	0.22	0.65	✱ **0.05**	0.884	0.66	0.65	0.5
All cecids?	0.4	**0.045**	0.28	0.15	0.78	**0.04**	0.5
bark hardness after decomposition	0.197	0.75	0.77	0.54	**0.02**	0.76	0.07
wood hardness after decomposition	0.264	0.8	0.15	0.21	0.79	0.88	0.91
All no. colours	0.85	0.82	0.3	0.39	0.14	0.82	0.45
All pignmentation	0.27	0.91	0.95	0.19	**0.05**	0.91	0.5

Key:
bold = P <0.05
*and **bold** = P < 0.05 + slope > 0.1

Key: INITIAL = initial wood-slice conditions measured, EMERGENT = final conditions measured after decomposition VC = Vuilleminia commedens, Hf = Hypoxylon fuscum, Combi = VC + Hf. rY = distance between medullary rays (mm), diam = diameter (cm), hardn's = harndness (N/cm2), b;rk thikn's = bark thickness (mm),